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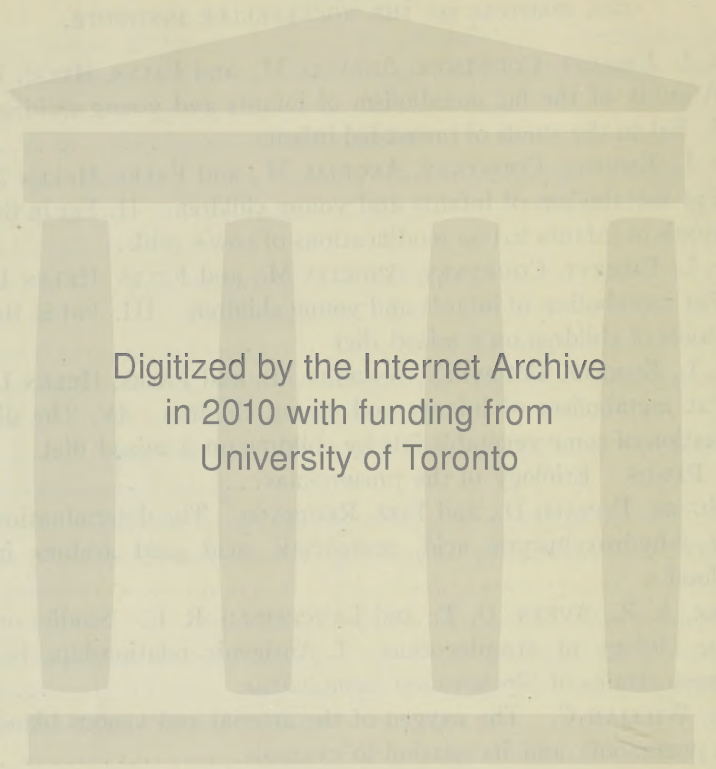
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## RELATION BETWEEN METAMORPHOSIS AND OTHER DEVELOPMENTAL PHENOMENA IN AMPHIBIANS.

By EDUARD UHLENHUTH.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 28, 1919.)

In 1833 von Schreibers<sup>1</sup> reported that in cold springs the larvæ of salamanders sometimes refrain from metamorphosis for a considerable length of time; upon dissecting such larvæ he found the remarkable fact that the sex organs had reached maturity before metamorphosis had taken place. Since that time, similar observations have been made repeatedly; but it was due especially to De Filippi's<sup>2</sup> discoveries, communicated in 1861, that the attention of biologists was directed to this phenomenon. De Filippi found a pond in the Formazza valley, near Andermatten, which was inhabited by numerous individuals of the species *Triton alpestris*. The majority of them had metamorphosed; but about forty were still in a larval condition and possessed gills. They were conspicuous not only by their unusually large size as compared with normal larvæ of this species, but also by the presence of mature sex organs. Soon after De Filippi's communication, Dumeril<sup>3</sup> reported his remarkable experiments on amphibians—probably the Mexican axolotl—in which it was demonstrated that the specimens in question were larvæ of the species *Ambystoma tigrinum* and not adult animals, and yet the larvæ not only possessed mature sex organs, but actually propagated in the laboratory while still in the larval condition.

These observations proved conclusively that the development of the sex organs in amphibians is independent of metamorphosis. Expressed in terms of Gudernatsch's<sup>4</sup> discoveries, according to which metamorphosis is caused by the action of definite substances, this would mean that the substances bringing about development of the sex organs are not identical with the substances causing metamorphosis. Recently the same condition has been found to prevail among

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<sup>1</sup> von Schreibers, *Oken's Isis*, 1833, 527.

<sup>2</sup> De Filippi, F., *Arch. per Zool. l'Anat. e Fisiol.*, Geneva, 1861, i, 219 (quoted by Wolterstorff).

<sup>3</sup> Dumeril, A., *Nouvelles Arch. Mus. Histoire Nat. Paris*, 1866, ii, 265; *Ann. sc. nat. Zool.*, 1867, vii, 229.

<sup>4</sup> Gudernatsch, J. F., *Arch. Entwicklungsmechn. Organ.*, 1912-13, xxxv, 457.



the tailless amphibians. Allen<sup>5</sup> has shown that extirpation of the thyroid in the larvæ of *Rana pipiens*, though it prevents metamorphosis, does not retard development of the sex organs, and Swingle's<sup>6</sup> experiments prove that feeding thyroid to tadpoles, though it enforces metamorphosis, does not accelerate development of the germ cells.

The fact that the germ cells in amphibians are independent of metamorphosis as regards their development has recently given rise on the part of Professor Allen<sup>5</sup> to renewed consideration of Weismann's hypothesis postulating the existence of a fundamental difference between the germ plasma and the somatic plasma, since the observations made in amphibia may be interpreted to mean that the germ plasma is independent of the somatic plasma. This interpretation, however, would be correct only if it could be shown that the independent behavior during development is peculiar only to the germ cells, and that consequently the soma as a unit is opposed to the germ cells as an independent unit. But we will show, in the present paper, that upon further analysis of amphibian development, it is found that the so called soma, in the Weismannian sense, does not exist; for it is possible to demonstrate that in salamanders this soma can be made to disintegrate into a number of organs which, like the sex organs, are independent of metamorphosis and, moreover, are independent of each other as regards their development. Furthermore, the nature of metamorphosis will have to be defined more accurately to mean not the development of the animal as a whole, but the development of certain organs of the amphibian organism. The remarkable feature in the development of amphibians is not the independence of the germ plasma from the somatic plasma, but the independence of various groups of organs from one another, due to the fact that the development of each of these groups is controlled by substances different from those controlling the other groups, and that each of these substances separately may be supplied to or withheld from the organism by the experimenter at will, either directly or by means of changing the environmental conditions.

<sup>5</sup> Allen, B. M., *J. Exp. Zool.*, 1917-18, xxiv, 499.

<sup>6</sup> Swingle, W. W., *J. Exp. Zool.*, 1917-18, xxiv, 521.

*Relation between the Reduction of the Gills, the First Skin Shedding, and Metamorphosis.*

It has become customary to apply the term metamorphosis to the processes which can be enforced by thyroid treatment. Though it is claimed frequently that in tadpoles the action of iodine can be defined accurately, it seems that certain phenomena have been included as a result of iodine action which in reality are not due directly to this action. It will be shown that the effect of iodine can be determined most accurately in salamanders, at least as regards two organs, the gills and the skin, while the development of several organs can be shown to be independent from the iodine mechanism.

In salamanders, two phenomena are most conspicuous during metamorphosis; the first shedding of the skin and the reduction of the gills.

In temperatures of from 20–30°C., the first shedding of the skin never takes more than 24 hours and frequently only from 2 to 5 hours. In lower temperatures it may take from 1 to several days. But in both cases the beginning and the end can be determined with accuracy if the animals are watched carefully. It is thus possible to observe accurately the time relation which exists between the first shedding of the skin and other developmental phenomena. While several of the latter phenomena have been observed to take place either before or after the first shedding of the skin, as will be discussed below, the time relation between the shedding of the skin and the reduction of the gills is constant and unchangeable. The condition of the gills (external in salamanders) varies greatly and is influenced much by the quantity of food available to the larvæ, as Powers<sup>7</sup> has stated. Well nourished larvæ possess large gills with long fringes; in poorly nourished animals the stems as well as the fringes become short. But in whatever state the larvæ may be kept, the gills are always characterized by the possession of stems as well as fringes. In metamorphosis, however, the gills are reduced to short stubs without fringes. The transition from the state with fringes into that without fringes is sudden and definite, and requires only a few hours.

<sup>7</sup> Powers, J. H., *Am. Nat.*, 1903, xxxvii, 385.

Many hundreds of salamander larvæ belonging to the species *Ambystoma maculatum*, *Ambystoma opacum*, *Ambystoma tigrinum*, and *Eurycea rubra* have been observed under the most varying conditions during metamorphosis; but in not one instance were the gills reduced to mere stubs without fringes before the shedding of the skin had taken place. This time relation is observable even under conditions of the greatest acceleration. Since it will become evident, and has already been pointed out above that a similarly constant succession in time between the two phenomena in question and other developmental phenomena does not exist, we are forced to assume that a relation exists between the shedding of the skin and the reduction of the gills, which does not exist between these phenomena and other developmental processes. Evidently both these phenomena must have a common cause, and, indeed conditions which retard or prevent the shedding of the skin also retard or prevent the reduction of the gills, while conditions which accelerate or enforce the shedding of the skin also accelerate or enforce the reduction of the gills. Moreover, it can be shown that both phenomena are enforced by the application of iodine and inhibited by the lack of iodine; and consequently they must be considered as constituting part of the amphibian metamorphosis. Numerous experiments have been carried out to test the action of iodine upon the shedding of the skin and the reduction of the gills; two are described below.

A series of six larvæ of *Ambystoma opacum* (W<sub>I</sub> 1917) were kept in ordinary tap water at approximately 25°C. and fed on earthworms. 5 weeks after hatching, the larvæ had attained a length of 29.6 mm. and were in an early larval stage. At this time they were placed in a 0.02 per cent solution of Bayer's iodothyryl in tap water. 8 or 9 days later, the skin was shed and the gills were reduced rapidly to stubs without fringes, while the control larvæ needed several weeks more to undergo the same changes. This effect of iodine upon skin shedding and the reduction of the gills was particularly conspicuous as the development of other organs, *e.g.* the legs, did not make any progress. Thus the phenomena of skin shedding and reduction of the gills surely are caused by the action of iodine, and constitute what we must call metamorphosis.



In a previous paper<sup>8</sup> it has been demonstrated that the feeding of the thymus gland to larvæ of *Ambystoma opacum*, *Ambystoma maculatum*, and *Ambystoma tigrinum* frequently retards and in rare cases even inhibits metamorphosis, and that this inhibitory action of the thymus diet is due to the fact that iodine is either completely absent from the thymus or present in amounts so small as to make it impossible for metamorphosis to take place at a normal time. In the thymus-fed larvæ not only the reduction of the gills but also the shedding of the skin is either retarded or completely inhibited, and yet other organs may develop normally and reach an almost adult condition. This proves that while the development of the latter organs is independent of the iodine action, both the skin shedding and the reduction of the gills cannot take place in the absence of iodine.

*Relation between Development of Skin Coloration and Metamorphosis.*

Among the organs whose development is independent of metamorphosis, the structures which give rise to the coloration of the skin are most conspicuous. Since the coloration of the skin is easily observable without killing the animal, the development of this character can be used with great advantage to demonstrate its relation to metamorphosis. The color development of the skin is most variable as regards its time relation to metamorphosis. This relation is subject to change under a number of different conditions; the development of the skin coloration is not produced by the substances causing metamorphosis, for it takes place without the action of iodine.

Though careful studies on the subject in question have not been carried out thus far, the remarkable fact that gill-bearing larvæ may exhibit the coloration of metamorphosed animals has been observed repeatedly. In 1891 von Bedriaga<sup>9</sup> described in detail the coloration of a number of larvæ of tailed batrachians and frequently emphasized in this paper that neotenous larvæ of the genus *Triton* may possess colors similar to those of metamorphosed animals. Similarly

<sup>8</sup> Uhlenhuth, E., *J. Gen. Physiol.*, 1918-19, i, 305, 473.

<sup>9</sup> von Bedriaga, J., *Zool. Anz.*, 1891, xiv, 295, 301, 317, 333, 349, 373, 397.

Wolterstorff<sup>10</sup> observed that the coloration continued to develop in urodelan larvæ whose metamorphosis had been checked; he mentioned that in this phenomenon we seem to deal with a noteworthy case of organic correlation. He probably thought, however,—erroneously as will be shown later on—that the development of the skin pigmentation is conditioned by the development of the sex organs, a correlation existing between these two organs. Later on Kammerer<sup>11</sup> observed a larva of *Salamandra maculosa* which had developed the skin pattern of the adult a year and a half before metamorphosis took place. Similar phenomena have been observed also among the larvæ of *Salientia*. Dickerson,<sup>12</sup> for instance, in a description of the metamorphosis of *Rana clamitans*, says: "The variation in the size, color, and markings of the changing tadpoles is very great. Most curious is the fact that some tadpoles show the lateral folds and the coloring of the adult male or female long before the change is completed, while others take on the frog form entire before the lateral folds are well developed or before the sexual coloring is evident."

The following experiments show that the development of the skin coloration cannot be prevented by the absence of iodine from the food, nor can it be enforced by the direct application of iodine; consequently it is independent from metamorphosis and may take place either before or after metamorphosis.

Four series of larvæ of *Ambystoma opacum*, all hatched from eggs of one female, were kept at approximately 25°C.; two series (*A* and *E*, 1916) were fed earthworms, the other two (*B* and *F*, 1916) were fed calf's thymus. In Series *A* and *E* the gray network of the adult commenced to develop as soon as the animals had metamorphosed (Figs.

FIGS. 1 to 4. Development of the adult coloration of the skin in worm-fed and thymus-fed animals of *Ambystoma opacum*. In worm-fed animals the gray network develops after metamorphosis (Figs. 1 to 3), in thymus-fed animals, the metamorphosis of which is retarded, it develops before metamorphosis (Fig. 4). Photographed from water color paintings made from the living specimens. Natural size.

<sup>10</sup> Wolterstorff, W., *Zool. Garten*, 1896, xxxvii, 327.

<sup>11</sup> Kammerer, P., *Arch. Entwicklungsmechn. Organ.*, 1904, xvii. 165.

<sup>12</sup> Dickerson, M. C., *The frog book*, New York, 1907, 205.



FIG. 1.



FIG. 2.

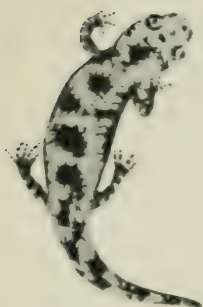


FIG. 3.

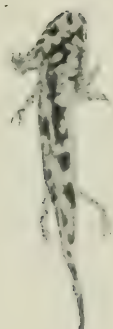


FIG. 4.

FIG. 1. *Opacum* 1916, A 4. Worm-fed metamorphosed animal, 2 days after metamorphosis; gray network visible on the snout; rows of larval spots still present.

FIG. 2. *Opacum* 1916, A 3. Worm-fed metamorphosed animal, 2 weeks and 3 days after metamorphosis. Gray network fully developed, but rows of larval spots still present on back.

FIG. 3. *Opacum* 1916, A 3. Worm-fed metamorphosed animal, 6 weeks after metamorphosis. Gray network fully developed, rows of larval spots have disappeared.

FIG. 4. *Opacum* 1916, F 3. Thymus-fed larva. Gray network fully developed; rows of larval spots have disappeared.



1 and 2), and was fully developed from 5 to 6 weeks after metamorphosis (Fig. 3). In Series *B* and *F* the gray network of the adult had started to develop before metamorphosis. In five larvæ whose metamorphosis was either checked completely or retarded to a considerable extent, the gray network reached a condition very similar to the adult condition as shown in Fig. 4.

These experiments have been repeated several times and have always furnished the same result; namely, that the development of the adult coloration can proceed in the absence of iodine, and can take place before metamorphosis has been accomplished. Moreover, in the above mentioned larvæ the sex organs were not in a mature condition; the development of the skin color is independent not only of metamorphosis but also of the development of the sex organs.

On the other hand, iodine treatment though it leads rapidly to metamorphosis does not advance the development of the skin coloration. This was shown most strikingly in the Series *Opacum* 1917, *W<sub>I</sub>*. In these larvæ the iodothyryn treatment enforced metamorphosis from 8 to 9 days after the beginning of the experiment; the small and little developed larvæ shed the skin and reduced the gills rapidly. The coloration of the skin, however, which at this time was that of an early larval stage, did not change at all under the influence of the iodine; consequently metamorphosed animals resulted with the color of young larvæ.

Similar experiments were conducted with the species *Ambystoma maculatum*. If the larvæ were fed on earthworms and kept at 25°C., the isolated yellow spots developed from the yellow network after metamorphosis; if they were fed on thymus and kept at 25°C., metamorphosis was retarded, but the development of the yellow spots proceeded notwithstanding.

Evidently the development of the structures leading to the coloration of the skin are caused by substances which are not identical with the substances causing metamorphosis, nor are they identical with the substances causing development of the sex organs. So far then, we have found three groups of organs, each of which is controlled by a different substance, or rather a different set of substances, as will be discussed below.

*Relation between Development of Legs and Metamorphosis.*

The statement is usually made that in tadpoles iodine enforces development of the fore limbs and that consequently the limb development of amphibians is part of the amphibian metamorphosis. We have observed that in salamanders the development of the limbs, the fore limbs as well as the hind limbs, is completely independent of metamorphosis; it is not in any way caused or even influenced by the iodine mechanism. On the one hand, the legs of thymus-fed animals develop at the same rate and to the same degree as in worm-fed control larvæ. On the other hand, larvæ subjected to iodine treatment do not show any precocious development of their limbs. In Series *Opacum* 1917, W<sub>I</sub> (described above), the hind limbs had developed only four toes when the larvæ were exposed to the iodothyrim solution. Though the iodothyrim, as mentioned above, caused rapid shedding of the skin and reduction of the gills to stubs without fringes, it did not bring about the development of the fifth toe of the hind limbs; thus metamorphosed salamanders resulted with only four toes on the hind limbs. Hence development of the legs is not caused by iodine, but by substances different from those which control metamorphosis.

In accordance with this, the salamander larvæ develop their legs normally if they are deprived of their thyroids even as early as the stage just before the beginning of the blood circulation; for Hoskins and Morris<sup>13</sup> mention that larvæ of *Ambystoma punctatum* after thyroid removal behave like the controls, except that they do not grow so well; but they do not mention that thyroidectomy prevented the legs from developing.

From this experiment it is quite evident that in contradistinction to what has been observed in frog tadpoles, in the larvæ of the caudate amphibians, leg development and metamorphosis are subject to two different factors, and that, consequently, leg development can occur even if no metamorphosis takes place, or may not occur even if metamorphosis takes place.

<sup>13</sup> Hoskins, E. R., and Morris, M., *Proc. Soc. Exp. Biol. and Med.*, 1916-17, xiv. 74.

It is remarkable, however, that in two so closely related groups of animals as the caudate and tailless batrachians, the same process should be caused by so different a mechanism. It is difficult to refute the suspicion that in the *Salientia* the development of the limbs may be initiated only indirectly by the iodine action, perhaps on account of some incidental anatomical structure which first must be broken down by the autolytic action of the iodine. And, in fact, there exists such a structure in the *Salientia*, which may be in the way of the development of the fore limbs at least. It is well known that in the frogs and toads the fore legs begin to develop long before they become visible externally; they develop enclosed in the gill chambers and covered by the skin of the body. Would it not be possible that the autolytic changes of the skin which finally lead to the first shedding of the skin and which are actually effected by the iodine action, must first be initiated by the iodine in order that the legs can be freed and full development can take place in the tadpoles? In this connection the experiments which Lenhart<sup>14</sup> performed on tadpoles in order to test the action of thyroid glands of varying iodine content may be mentioned. He noticed that with increasing iodine content the absorption of the tail takes place more and more quickly while differentiation, on the contrary, becomes less, taking the development of the limbs as expression of the differentiation. This result can be understood only if we assume that the autolytic processes alone leading to absorption of the tail, shedding of the skin, and metamorphosis, are caused directly by the action of the iodine. If the time between the beginning of the autolytic processes and their final result, the shedding of the skin, is long enough, the legs have time to develop after they have been freed, before metamorphosis takes place. But if by means of strong iodine concentration this interval becomes much shortened, the legs have no time to differentiate before metamorphosis occurs, the iodine, the concentration of which was increased artificially, acting more strongly than the substances causing leg development, which were not increased artificially.

That leg development is not a part of the amphibian metamorphosis is also demonstrated by the fact that in certain amphibians,

<sup>14</sup> Lenhart, C. H., *J. Exp. Med.*, 1915, xxii, 739.



such as the cæcilians, legs are completely absent in the adult form and yet a normal metamorphosis takes place in these amphibians.

There are still other organs the development of which does not seem to be caused by the iodine action in metamorphosis. It has been observed by Cope<sup>15</sup> and confirmed later by Powers<sup>16</sup> that in *Ambystoma tigrinum* under certain conditions metamorphosis may take place without the tongue and the larval arch of the palatal teeth developing the adult condition. From this may result metamorphosed animals without tongue and with the larval arch of the palatal teeth persisting. The substances causing metamorphosis apparently do not influence the development of the two organs in question. Thus it seems that at least six chemical mechanisms are present in the amphibian organism, one for metamorphosis, one for the skin coloration of the adult, one for the sex organs, one for the legs, one for the tongue, and one for the palatal teeth. The remarkable feature in the amphibian development is that each of these mechanisms can act independently without influencing the others; the organism as a whole, as an organic individuality, seems to have no control over the harmonic development of these groups of organs, since the chemical mechanisms separately respond to conditions which are not located within but without the organism. We will see now that one of these conditions is the temperature, which affects the rate of some of these mechanisms in a different way and consequently brings about unequal development of various parts of the body.

*Influence of Temperature upon Metamorphosis and Coloration of  
Ambystoma tigrinum.*

If the coloration of the skin is caused by substances different from those which cause the amphibian metamorphosis, we can understand the influence of temperature upon the coloration of *Ambystoma tigrinum* as observed in the following experiments.

Two sets of larvæ of *Ambystoma tigrinum*, *Tigrinum* 1917, Series S and U, were kept at approximately 25° and 15°C. respectively; other-

<sup>15</sup> Cope, E. D., The batrachia of North America, Washington, 1889, 73. (*Bull. U. S. Nat. Mus.*, No. 34).

<sup>16</sup> Powers, J. H., *Univ. Studies Univ. Nebraska*, 1907, vii, 197.

wise they were kept under exactly like conditions. The eggs from which they hatched in the laboratory were collected in Syosset, Long Island, in the spring of 1917; they were all taken from one single egg mass and were consequently laid by the same female. Soon after hatching the larvæ of both sets were fed on worms, which were used as food throughout the whole experiment. Each set consisted of six specimens; five of these metamorphosed in Series *S* and four in Series *U*. In Series *S*, metamorphosis took place 11 weeks and 6 days after hatching, in *U*, 22 weeks and 2 days after hatching.

In both series the color of the larvæ before metamorphosis was an even greenish black.

In Series *S* numerous yellowish spots of small size became visible all over the back as soon as the larvæ had shed the skin (Fig. 5). In Series *U* the larval color was retained after metamorphosis and no spots appeared; metamorphosed animals with a larval coloration resulted (Fig. 6).

In Series *S* the yellow spots soon began to increase considerably in size; in Series *U* no yellowish spots appeared before the 2nd week after metamorphosis, when a small number of tiny sepia colored specks made their appearance.

In Series *S* the yellowish spots had increased so much in size several weeks after metamorphosis that they became confluent here and there forming yellow blotches; no further changes took place in Series *U*. Fig. 7 shows an animal of Series *S* 13 weeks and 2 days after metamorphosis; the spots have fused to form blotches and bands of yellowish color. Fig. 8 shows an animal of Series *U*, 22 weeks and 3 days after metamorphosis; the spots are still small and few in number.

The final coloration of the adults is shown in Figs. 9 and 10. In Fig. 9 an animal of Series *S* is shown 33 weeks and 2 days after metamorphosis; very little of the black background is left, most of it being filled in by the large yellowish bands; in the animals kept at low temperature just the opposite occurs, as is shown in Fig. 10, 50 weeks and 5 days after metamorphosis. The number and size of the yellowish spots have increased only slightly.

These experiments show that the occurrence of banded and spotted individuals in *Ambystoma tigrinum* is not due to the existence of two different races, since in our experiments all animals were derived



FIG. 5.



FIG. 6.

FIGS. 5 to 10. In *Ambystoma tigrinum* the development of the yellowish spots is more retarded at low temperature than metamorphosis, and the development of the yellowish bands is completely inhibited. Fig. 6 photographed from live specimen. Fig. 10 photographed from water color painting made from preserved specimen. Rest of figures photographed from water color paintings made from live specimens. Natural size.

FIG. 5. *Tigrinum* 1917, S 3. 25°C., 12 days after metamorphosis. Numerous small yellowish spots.

FIG. 6. *Tigrinum* 1917, U 4. 15°C., 11 days after metamorphosis, larval coloration. No spots.

from one female. Furthermore, the difference in coloration as observed here is not due to the influence of light but to the influence of temperature. The action of the temperature is manifested in two ways: first, in low temperature the development of the yellow spots





FIG. 7.

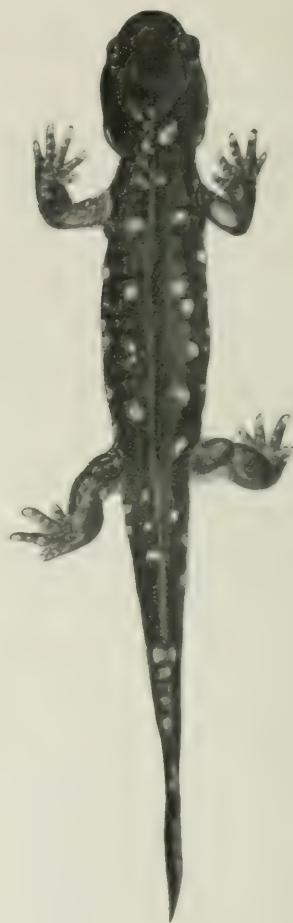


FIG. 8.

FIG. 7. *Tigrinum* 1917. S 4. 25°C., 13 weeks and 2 days after metamorphosis. Yellowish spots fused to yellow blotches and bands.

FIG. 8. *Tigrinum*. 1917. U 4. 15°C., 22 weeks and 3 days after metamorphosis. Few small yellowish spots; no bands.



FIG. 9.

FIG. 9. *Tigrinum* 1917, S 4. 25°C., 32 weeks and 2 days after metamorphosis. Only little is left of the dark background, most of the space being taken up by the yellowish bands.



FIG. 10.

FIG. 10. *Tigrinum* 1917, U 4. 15°C., 50 weeks and 5 days after metamorphosis. Only small yellowish spots present; no bands have developed

is more retarded than metamorphosis; second, the development of yellow bands is inhibited permanently in low temperature.

The first phenomenon can be explained as follows: In a previous paper it was shown that one of the substances causing metamorphosis, the excretor substance, is evolved during chemical processes, the rate of which depends on the rate of the processes leading to growth. Since the temperature coefficient of the excretor-forming processes is greater than that of the growth processes, the formation of excretor substance is decreased more than the rate of growth if the larvæ are kept at low temperature, and consequently the relation between size and metamorphosis is changed, the larvæ being larger at the time of metamorphosis when kept at low temperature than when kept at high temperature. Since the temperature coefficients of different physiological processes are frequently different, we might expect that the temperature coefficients of the processes causing metamorphosis are different from those of the processes causing development of the structures which give rise to the skin coloration of the metamorphosed animal. If this were so, we should expect that the time relation between metamorphosis and the development of the skin color should be changed not only by feeding or omitting substances which cause precocious metamorphosis but also by keeping the animals at low temperatures.

This is actually the case with the development of the yellowish spots in *Ambystoma tigrinum*. The retardation of the appearance of the yellowish spots as compared with metamorphosis can be explained if we assume that the chemical processes causing the development of the spots have a higher temperature coefficient than the chemical processes causing metamorphosis.

In order to understand the second phenomenon, the inhibition of the development of the yellowish bands, we shall again refer to metamorphosis.

One of the cases of metamorphosis most difficult to explain is the occurrence of a perennibranchiate form in the species *Ambystoma tigrinum* in certain localities. On the basis of the results so far obtained in our experiments the perennibranchiate form of the *Ambystoma tigrinum* can be explained tentatively as follows.



Two reasons may be assumed to account for the inhibition of metamorphosis in *Ambystoma tigrinum*. First, it is possible that with progressing age certain changes occur in the organism which abolish the susceptibility of the larvæ to the action of iodine; but experiments have shown that even very old larvæ can be forced to metamorphose by direct application of iodothylin.

Second, it is possible that with progressing age certain changes occur in the thyroid gland which make this gland progressively less susceptible to the action of the excretor substance. By this assumption the problem can be solved. The localities where the perenni-branchiate form of *Ambystoma tigrinum* occurs are all located high in the mountains and consequently the temperature of the water in these lakes is very low. As in other cases it is possible that the temperature coefficient of the thyroid change is lower than that of the excretor formation, and that for this reason lowering of the temperature decreases the rate of the excretor formation more than it decreases the rate of the thyroid change. Thus at the time when the quantity of excretor required under normal conditions to induce thyroid excretion would be evolved, the thyroid would be in a state where this quantity would not any longer be sufficient to affect excretion. If the rate of the thyroid change which makes this organ less and less sensitive to the action of the excretor, and the rate of the excretor formation would be constant under these conditions, the difference between the quantities of the two factors would increase progressively, and metamorphosis would become more and more impossible.

The development of the yellow bands of *Ambystoma tigrinum* in low temperature is indeed very similar to the metamorphosis of the same species, both becoming inhibited permanently by low temperature; the only difference is that inhibition of the development of the yellow bands can be effected at a temperature somewhat higher than that at which metamorphosis can be inhibited. From this similarity we may conclude that the development of yellow bands in *Ambystoma tigrinum* is due to an interaction of factors similar to but not identical with those involved in metamorphosis. Possibly one of these factors is a structure which loses in susceptibility towards a

certain substance with progressing age, the temperature coefficient of that change being lower than that of the processes which produce the substance in question.

It is possible that mechanisms similar to the mechanism involved in the development of the skin color are responsible also for the development of the tongue and palatal teeth, both phenomena being checked definitely by low temperature, as pointed out by Cope<sup>15</sup> and Powers.<sup>16</sup>

#### DISCUSSION.

In previous experiments<sup>17</sup> it has been shown, by grafting organs from one larva to a larva of different age, that the development of certain stages of the eye and of the skin pigmentation is caused, like the processes of metamorphosis, by substances which are not produced in the organs themselves but reach them through the blood circulation. In this paper further proof of the existence of such substances has been found in experiments made by a different method. But at the same time it has been shown here that the substances which are causing development of these organs are not identical with the agents causing metamorphosis and consequently the development of these organs cannot be included under metamorphosis. It is certain that in the salamanders examined at least five groups of organs, the structures leading to pigmentation of the skin, sex organs, legs, tongue, and palatal teeth, are independent of each other and of metamorphosis. It is probable that at least six different chemical mechanisms exist in the amphibian organism each of them for another group of organs. Consequently it should be possible to change the succession in time of these developmental phenomena by feeding directly substances which contain or lack the agent for only one of them; this can be actually accomplished in the case of metamorphosis and development of the skin color by iodine treatment or thymus feeding. But the same result should be accomplished by keeping the larvæ in different temperatures, since the temperature coefficients are different for the chemical reactions constituting each of these mechanisms. This has

<sup>17</sup> Uhlenhuth, E., *Arch. Entwicklungsmechn. Organ.*, 1913, xxxvi, 211; *Arch. vergl. Ophthalm.*, 1913, iii, 343; *Proc. Soc. Exp. Biol. and Med.*, 1917, xiv, 88; *J. Exp. Zool.*, 1917-18, xxiv, 237.

been shown to be actually the case for metamorphosis and coloration in *Ambystoma tigrinum*, and probably the same cause is responsible for the production of metamorphosed animals without tongue and with a larval arch of palatal teeth.

It is probable that two factors are causing metamorphosis, iodine and excretor substance. A third factor is probably involved in inhibition of metamorphosis at low temperature; namely, a change of the thyroid gland, making this organ progressively less susceptible to the excretor action, with progressing age. The inhibition of the development of the yellow bands at low temperature in the species *Ambystoma tigrinum* is so similar to the inhibition of metamorphosis by low temperature in the same species as to suggest that the mechanism causing this coloration is composed also of a number of factors interrelated in a way similar to that of the factors for metamorphosis. It would be interesting to find out whether one of these factors, as in metamorphosis, is an internal secretion.

The experiments reported here show that the independent development of the germ cells cannot be used to postulate an independent position of the germ plasma as compared with the somatic plasma, since other groups of organs which so far as known are not composed of germ plasma behave like the germ cells. The fact that in salamanders different groups of organs are evolved by the action of different substances seems to agree well with similar phenomena in plants, since it has been shown that the different organs of the plant are caused to develop separately and apparently without the control of what may be called an organic individuality, by the action of different substances, special substances, for instance, serving for development of leaves and roots.<sup>18</sup>

#### SUMMARY.

1. The difference in time existing between the first shedding of the skin and the reduction of the gills to mere stubs without fringes is constant and unchangeable, which indicates that the fundamental cause for both is a common one.

2. This common cause is the action of iodine, and consequently both phenomena constitute, or at least are part of, the metamorphosis of the salamanders.

<sup>18</sup> Loeb, J., *Bot. Gaz.*, 1915, lx, 249; 1916, lxii, 293; 1917, lxiii, 25.



3. The development of the adult skin coloration and of the legs may take place either before or after metamorphosis. Iodine cannot enforce either of these phenomena.

4. The same is true of the development of the sex organs.

5. Development of the tongue and palatal teeth can be checked even in animals in which metamorphosis takes place.

6. Consequently development of the skin coloration, as well as development of the legs, sex organs, tongue, and palatal teeth are all caused by substances not identical with the substances causing metamorphosis and, since they are also all independent of each other in their development, it is probable that special chemical mechanisms exist for the development of each one of these six groups of organs.

7. This assumption is also supported by the fact that the order of development in several of these organ pairs can be changed by a difference in temperature, which would indicate that the development of each of these groups of organs is caused by chemical reactions with different temperature coefficients.

8. That the germ cells can develop in amphibians either before or after metamorphosis does not mean that the germ plasma is opposed as a unit to the somatic plasma, since other organs which are believed to be part of the somatic plasma behave in this respect like the germ cells.

9. The noteworthy feature of the amphibian metamorphosis is that instead of being controlled and kept in harmony by the organic individual the development of at least six groups of organs is controlled separately by the action of probably six different chemical mechanisms, each of which can be stopped or enforced independently either by directly supplying the substances required or by causing an increased formation within the body by suitable temperatures.

## THE AGGLUTINATION REACTIONS OF THE MORGAN BACILLUS NO. 1.

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(Received for publication, March 31, 1919.)

In the course of an investigation on the problem of soil pollution, bacilli having the cultural properties of the Morgan bacillus No. 1 were isolated from three polluted wells. The epidemiological significance of these findings is apparent provided that this organism really bears the relation to infectious diarrhea claimed for it by its discoverer. It was not feasible to enter into the question of etiology, but it has seemed desirable to compare the strains isolated from the polluted wells with some obtained from human sources. The resulting observations are of interest because they throw some light on the nature of this bacillus, which has been the subject of a great deal of discussion.

This organism was first described by Morgan in 1906. It belongs to the colon-typhoid group, but is peculiar in that it ferments only the hexoses with the production of gas. In the first epidemic of infectious diarrhea in which Morgan observed this organism he succeeded in isolating it from the stools of 40 out of 112 patients. In subsequent epidemics in England Morgan and his coworkers as well as Ross, Alexander, Lewis, and others, paid special attention to this type of bacillus. The reported findings are greatly at variance. Morgan isolated it in 54 per cent of the cases in 1905, 56 per cent in 1906, 16 per cent in 1907, and 53 per cent in 1908. Ross, Orr, and Alexander found this bacillus together with other non-lactose-fermenting bacteria, but claim that no one type was conspicuous. Alexander reports that during 1911 and 1912 the Morgan bacillus No. 1 was found in the stools of 4 per cent of normal and in 13.4 per cent of sick children examined. Lewis, on the other hand, in 1910 failed to isolate the organism from the stools of normal children, whereas he found it in 30 per cent of cases suffering from acute diarrhea. In 1911 he isolated the bacillus from 17 per cent of normal children and 70 per cent of the cases of diarrhea.

This peculiar organism has also been cultivated from sources other than human. Morgan and Ledingham isolated it from one cow out of eighteen examined; Lewis obtained it from five out of twenty mice. Cole isolated two strains from the intestinal contents of roaches.

The pathogenicity of the strains is variable. Both Morgan and Lewis proved that some strains were fatal for rats, mice, and rabbits when given *per os*. Neither Morgan nor Lewis was able to demonstrate the presence of specific antibodies in the sera of children from whom the bacilli were isolated.

The agglutination reactions of a large number of strains against different sera were reported by Lewis in 1912. He tested 242 strains from 167 cases, including those isolated from normal individuals, mice, cow, etc. against 16 sera. He found three main groups containing respectively 48, 17, and 8 per cent of the strains. There was no correlation between agglutination group and source. 11.5 per cent of the strains did not agglutinate with any of the sera. Lewis did not carry out any absorption tests.

It is evident from this brief review that the etiologic significance of the Morgan bacillus is by no means established. The work of Flexner and his coworkers, TenBroeck and Norbury, and Smillie leaves little room for doubt that in this country infectious diarrhea of infants is mainly caused by the dysentery bacilli. There remains, however, the possibility that the Morgan bacillus does play a minor part, since reports of its isolation frequently appear in the literature.

#### EXPERIMENTAL.

*Strains Studied.*—The total number of strains tested was small but representative. In addition to the three strains isolated from polluted water there were fourteen others, obtained from a variety of sources. Three came from the Lister Institute, England; two were from the Institute of Public Health, Canada, and were originally sent there by Captain Fidler from Mesopotamia. Three cultures were isolated in this country from stools of infants suffering from acute diarrhea. The others were either stock strains of unknown origin or recently isolated from other sources. Table I gives the number, source, and, when known, the year of isolation of the cultures used.

*Cultural Characters.*—Morphologically and culturally all the strains were identical and corresponded with the original description given by Morgan. They were all motile, Gram-negative bacilli, resembling the other members of the typhoid-dysentery group. They fermented the hexoses (dextrose, levulose, and galactose) with the production of acid and gas, but failed to ferment any of the other fermentable substances tested (mannite, maltose, lactose, saccharose, xylose,

arabinose, rhamnose, salicin, and dulcitol). In semisolid agar containing glycerol and Andrade indicator they produced acid in the upper (aerobic) and not in the lower (anaerobic) part of the tube. All of them produced an abundance of indol in 24 to 48 hours in beef infusion broth. Gelatin was not liquefied. In their behavior toward the triphenylamine dyes they were more akin to the typhoid than to the dysentery bacilli.

TABLE I.  
*List of Cultures Used.*

Strain No.	Source.
M38a	Lister Institute, England. Origin not given.
M45a	" " " " " "
M33	" " " " " "
M.J.	Institute of Public Health, Canada. Obtained from Captain Fidler, Mesopotamia.
M.D.	Institute of Public Health, Canada. Obtained from Captain Fidler, Mesopotamia.
139	Stock strain from the American Museum of Natural History, isolated from water.
T17	Isolated, 1917 from the stool of a normal individual.
57	" 1917 " polluted well water.
318	" 1917 " " " "
25	" 1918 " " " "
M.R.	" 1916 " the intestine of a roach (Cole).
M.RX	" 1916 " " " " " " "
586	" 1915 " " stool of an infant (diarrhea).
692	" 1915 " " " " " " "
M.K.	Received from Professor Kendall. Origin unknown.
M.T.	" " Dr. TenBroeck. Isolated Aug., 1914, from a case of infant diarrhea in Boston.
S2	Isolated, 1917, from polluted soil.

*Pathogenicity.*—The pathogenic property of some of the strains was tested by feeding them to white mice and rats. The method of feeding was as follows: Veal broth containing 10 gm. of fresh veal to 100 cc. of the broth was used. The inoculated tubes were incubated for 48 hours at 37°C., and 10 cc. of these cultures fed to each animal by soaking the bread fed to them. Some of the animals were fed 10 cc. of culture on 2 consecutive days. None of the mice or rats became ill, although the bacilli were abundant in the feces for



several days after the feeding. The bacilli usually disappeared from the feces within 5 to 10 days after the feeding. Strains 692 and 586, most recently isolated from cases of acute diarrhea, and Strains 57, 318, and 139, obtained from polluted water, were employed in these tests.

*Agglutination Reactions.*—Agglutination tests were made with nine sera. The sera were prepared by injecting saline suspensions of

TABLE II.

*Agglutination Reactions of Morgan Bacilli in Different Sera.*

Strain No.	Agglutination with Serum.								
	692	586	139	318	M. K.	T 17	33	45	38
M38a	—	—	—	—	—	1,280*	320	2,560	2,560
M45a	—	—	—	—	—	2,560	640	2,560	640
M33	—	—	—	—	—	1,280	1,280	1,280	2,560
M.J.	1,280	—	80	—	—	—	—	—	—
M.D.	—	—	—	—	2,560	2,560	—	40	80
139	—	—	1,280	—	—	—	—	—	—
T17	—	—	—	—	320	2,560	—	2,560	640
57	—	40	—	80	—	—	40	—	—
318	—	—	—	2,560	—	—	—	—	—
25	—	—	1,280	—	—	—	—	—	—
M.R.	—	—	40	—	80	1,280	—	—	—
M.RX	—	—	—	—	—	—	—	—	160
692	1,280	—	—	—	—	—	—	—	—
586	—	1,280	—	—	—	—	—	—	80
M.K.	—	—	—	—	2,560	2,560	—	640	—
M.T.	—	—	—	—	—	—	160	—	—
S2	—	1,280	—	—	—	—	—	—	—

\* The numbers indicate the highest dilution at which agglutination occurred.

24 hour growths on agar slants. Five injections at 3 day intervals were usually given. The results are summarized in Table II. The numbers in the table indicate the highest dilution in which agglutination occurred. It is evident at a glance that the strains are highly diversified antigenically. The seventeen strains fall into six distinct groups.

*Absorption Tests.*—The heterogeneity of these strains is more strikingly shown by the absorption tests. Cultures T17 and M.K., for

example, cross-agglutinated in high dilutions, but their respective sera behaved differently towards the other strains. Absorption tests

TABLE III.

*Absorption of Agglutinins from Sera Produced against Various Strains of Morgan Bacilli.*

No. of agglutinating culture.	Serum 38 absorbed with.														
	Culture 38.					Culture 33.					Culture 45.				
	80	160	320	640	1,280	80	160	320	640	1,280	80	160	320	640	1,280
38	++	+	-	-	-	++	++	++	+	+	++	++	++	++	+
33	+	=	-	-	-	++	++	+	-	-	++	++	++	+	-
45	-	-	-	-	-	+	=	-	-	-	++	++	+	-	-
T17	-	-	-	-	-	+	=	-	-	-	++	++	=	-	-
	Serum 45 absorbed with.														
	Culture 45.					Culture T17.					Culture 38.				
	80	160	320	640	1,280	80	160	320	640	1,280	80	160	320	640	1,280
38	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
45	+	-	-	-	-	=	-	-	-	-	++	++	++	+	-
T17	+	-	-	-	-	=	-	-	-	-	++	++	++	+	-
	Serum 33 absorbed with.														
	Culture 33.					Culture 45.									
	80	160	320	640	1,280	80	160	320	640	1,280	80	160	320	640	1,280
33	-	-	-	-	-	++	++	++	++	++					
45	-	-	-	-	-	-	-	-	-	-					
	Serum T17 absorbed with.														
	Culture T17.					Culture M. K.									
	80	160	320	640	1,280	80	160	320	640	1,280	80	160	320	640	1,280
T17		-	-	-	-	++	++	++	++	++					
M.K.		-	-	-	-	-	-	-	-	-					
	Serum M. K. absorbed with.														
	Culture M. K.					Culture T17.									
	80	160	320	640	1,280	80	160	320	640	1,280	80	160	320	640	1,280
T17	-	-	-	-	-	+	=	-	-	-					
M.K.	+	-	-	-	-	++	++	++	++	++					

showed that they were distinct types. Similarly, the sera produced against the English strains M38a, M45a, and M33, cross-agglutinated the heterologous strains in high dilution, but absorption tests

showed that the three organisms were antigenically different. The seventeen strains are thus separated into ten distinct antigenic types without any correlation as to source. The results of the absorption tests are given in Table III.

#### SUMMARY AND CONCLUSION.

The study of the cultural and agglutinating reactions of seventeen strains of Morgan bacilli is reported. The cultures were obtained from different sources. Culturally all the strains were identical. Antigenically they were highly diversified. The sera produced against some strains have marked agglutinating power for other strains, but absorption tests showed that the cross-agglutinations were often due to group agglutinins. While no conclusion can be drawn regarding the pathogenic significance of this bacillus, the wide diversities of antigenic properties raise the question as to the specific relationship of the various cultures met with as well as their relation to a definite class of pathological processes in man.

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## ETIOLOGY OF YELLOW FEVER.

### I. SYMPTOMATOLOGY AND PATHOLOGICAL FINDINGS OF THE YELLOW FEVER PREVALENT IN GUAYAQUIL.

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PLATES 31 TO 34.

(Received for publication, March 24, 1919.)

In this paper it is proposed to describe the clinical features and pathological changes observed in yellow fever cases admitted to the Yellow Fever Hospital in Guayaquil.<sup>1</sup> It may be stated at the outset that in an analysis of 172 cases no clinical or pathological facts were brought to light which had not been described by previous students of yellow fever in Guayaquil<sup>2</sup> or elsewhere. The yellow fever cases occurring in Guayaquil present no special feature; the disease is classic in all its aspects. For the sake of clearness the general clinical features and the individual symptoms of the disease will be described separately.

<sup>1</sup> This hospital is under the direction of Dr. Wenceslao Pareja. Dr. Pareja not only pointed out many interesting clinical features but also performed autopsies for the members of the Yellow Fever Commission during their sojourn in Guayaquil. I am greatly indebted to Dr. Pareja for his cooperation in my investigations and to Dr. Charles Elliott of the Commission for permission to use some of his clinical notes made on about half the total number of cases coming under my observation during my stay. The Yellow Fever Commission of the International Health Board was composed of Dr. Arthur I. Kendall, Dr. Charles A. Elliott, and Mr. Herman Edward Redenbaugh of Northwestern University Medical School, Chicago; Dr. Mario Lebreto of Las Animas Hospital, Havana, Cuba; and Dr. Hideyo Noguchi of The Rockefeller Institute for Medical Research, New York.

<sup>2</sup> Strong, R. P., Tyzzer, E. E., Brues, C. T., Sellards, A. W., and Gastiaboru, J. C., Harvard School of Tropical Medicine, Report of the first expedition to South America, 1913, Cambridge, 1915, 180-200.



*Clinical Features.**General Symptomatology.*

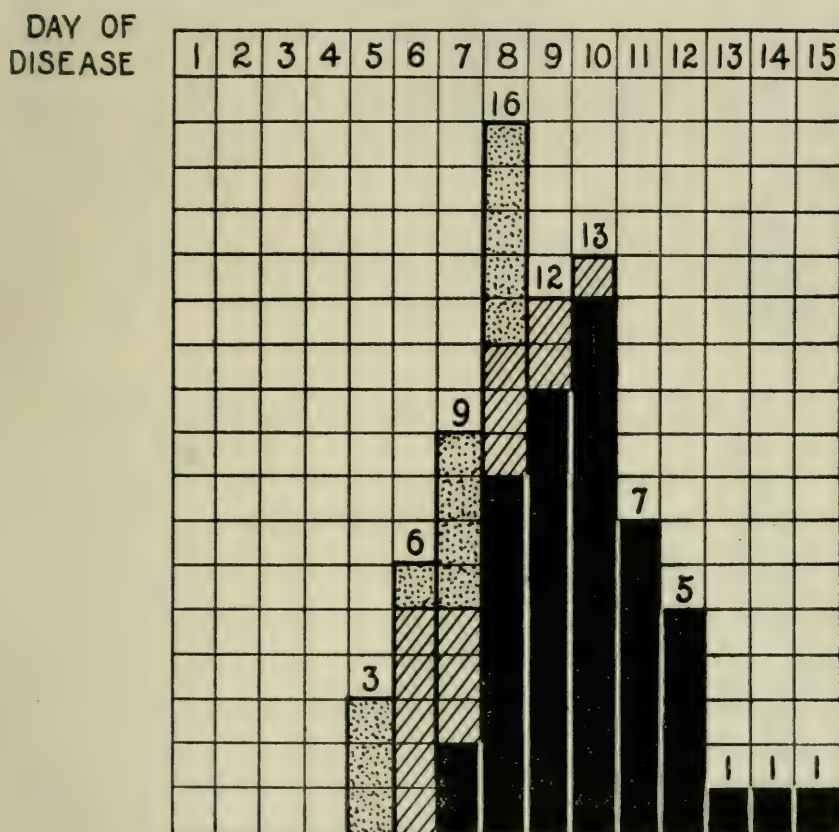
The incubation period varies from 3 to 6 days. It is difficult to determine the maximum. There may be prodromal symptoms for 1 or 2 days, but the onset is usually sudden, ushered in by fever, with or without chills. The patient feels gravely ill and in most cases goes to bed at once, with severe headache, pains in the loins, and anorexia; myalgia of the back, neck, and limbs is often intense, especially on pressure. Nausea and vomiting are frequently present. Insomnia and prostration follow. There is almost always tenderness in the epigastric region which gradually becomes aggravated as the disease progresses. The tongue has a heavy white coat with red tip and edge; later it may become brownish and dry. A peculiar, cadaveric-like odor emanates from the mouth. The gums are congested, swollen, and show a tendency to bleed on pressure. There is great thirst. The conjunctivæ are markedly suffused, becoming yellowish on the 2nd to 3rd day, sometimes with a few ecchymoses on the 6th to 7th day. The icterus of the conjunctivæ increases in the several days following and may persist 2 to 3 weeks in severe cases, although in milder ones it disappears in about 7 days. The black vomitus may occur on the 1st day or as late as several days after onset, or it may only be found in the stomach at autopsy. The skin is usually dry and icteric, and the patient suffers from intense epigastric pain. The gums may bleed profusely at this stage. The urine is reduced in volume; in many cases there is anuria for a day; the urine is dark, greenish, or brown, with abundant albumin and casts. Epistaxis occurs in many cases. Hiccoughs and other nervous symptoms (delirium, coma, convulsions) due to uremia and cholemia are frequent. Death may occur between the 4th and 9th days, rarely earlier or later.

*Individual Symptoms.*

*Fever.*—The fever is very high for 1 to 2 days, reaching 39–41°C., then drops to about 38°C. and may persist from 3 to 8 days. On the average the temperature drops to 37° or even to 36°C. after 8 days and may continue to be subnormal for several days.

The relation between the temperature and the severity of the infection among those who recovered is interesting.

*Recovered Cases.*—There were 74 cases of yellow fever which could be analyzed. These cases came under our observation at different



TEXT-FIG. 1. 74 non-fatal cases analyzed according to defecrescence and severity of the disease. The light shaded area signifies mild, the cross-hatching moderately severe, and the solid black severe cases.

stages of the disease, ranging from the 2nd to the 8th day. The temperature in early cases (2nd, 3rd, 4th days) was usually very high, the majority being over  $40^{\circ}\text{C}.$ , while those admitted later (5th to 8th days) had a temperature of  $39^{\circ}\text{C}.$  or below. The abate-

ment of fever to normal, or  $37^{\circ}\text{C}$ ., occurred as convalescence was established. In the majority of instances in which the attack was mild or of moderate severity the temperature returned to normal on the 5th and 6th days. Those whose temperature returned to  $37^{\circ}\text{C}$ . on the 7th day were cases of moderate severity, though a few mild and severe cases had similar febrile reactions. The cases in which the temperature returned to  $37^{\circ}\text{C}$ . on the 8th, 9th, or 10th day were nearly all severe, and all those in which the normal was not reached until the 11th day or later were severe. The deduction from

TABLE I.

*Relation between Time of Defebrescence and Severity of the Disease.*

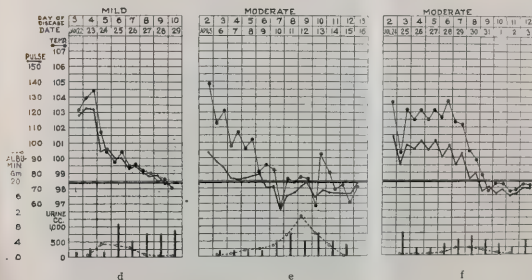
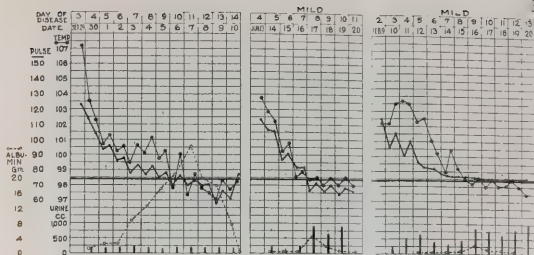
Day of disease when temperature reached $37^{\circ}\text{C}$ .	Mild.	Moderate.	Severe.	Total.
5th	3			3
6th	1	5		6
7th	4	3	2	9
8th	5	3	8	16
9th		2	10	12
10th		1	12	13
11th			7	7
12th			5	5
13th			1	1
14th			1	1
15th			1	1
Total.....	13	14	47	74

these observations is that the milder the attack the sooner the temperature returns to normal, and *vice versa*. In mild but undoubted cases of yellow fever the earliest date on which the normal has been attained is the 5th day of the disease (4 per cent). The bulk of the cases reached the normal on the 7th (12 per cent), 8th (20 per cent), 9th (16 per cent), or 10th (17 per cent) day. Text-fig. 1 and Table I show the distribution of cases from the standpoint of the time of defebrescence and severity of the disease.

It was found that in the cases which were admitted to the hospital from the 2nd, 3rd, or 4th day of the disease there are several instances in which the temperature registered higher the following



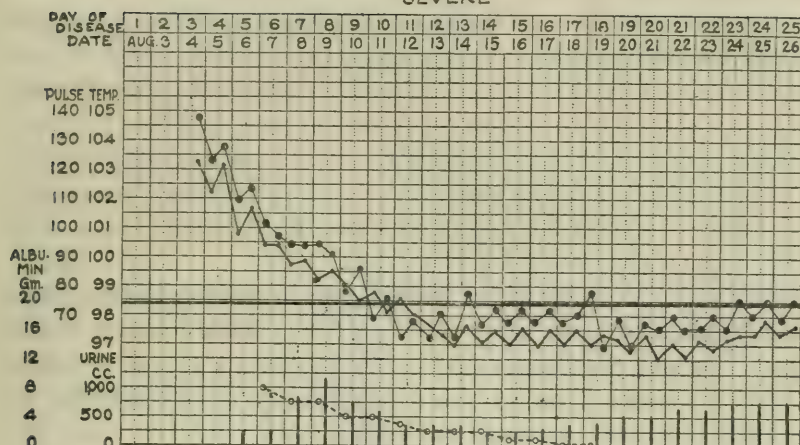




— — — Temperature.  
— — — Pulse.  
— — — Albumin in the urine.  
▮ Volume of urine for 24 hours.

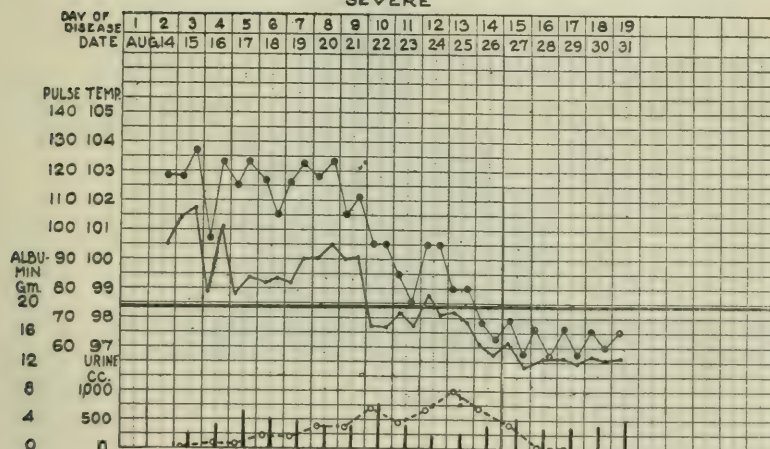
TEXT FIG. 2. a to f. Mild and moderate cases of yellow fever. (a) Case 35. Age 25 years. (b) Case 36. Age 21 years. (c) Case 37. Age 20 years. (d) Case 38. Age 20 years. (e) Case 39. Age 18 years. (f) Case 9. Age 19 years.

## SEVERE



a

## SEVERE



b

TEXT-FIG. 3, *a* and *b*. Severe cases of yellow fever. (*a*) Case 19. Age 25 years. (*b*) Case 8. Age 21 years.

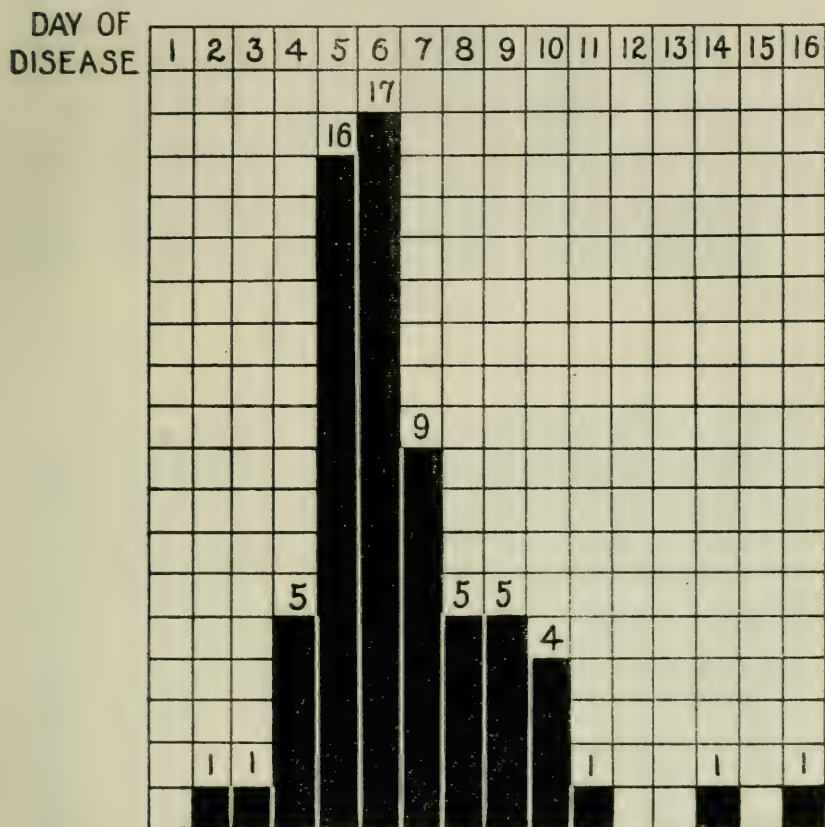
day than at the time of admission or that in some instances there was a distinct remission on the 2nd or 3rd day of the disease. Again in rare instances a relatively high fever ( $39-39.6^{\circ}\text{C}.$ ) lasted several days. The rapidity of lysis is somewhat variable in the different cases, but the drop in the curve is rather steep. When the temperature reaches  $37^{\circ}\text{C}.$  it usually goes further down, even as low as  $36^{\circ}\text{C}.$  within a few days and may remain subnormal for several days before it attains the normal permanently. Text-figs. 2, *a* to *f*, and 3, *a* and *b* illustrate these points.

*Fatal Cases.*—A study of the temperature in relation to fatal cases seemed important to an understanding of the clinical features of this disease. Among the records for the year (1918) I was able to utilize 66 cases of persons dying of yellow fever (Table II and Text-fig. 4). The bulk of deaths occurred on the 5th, 6th, and 7th days, but especially on the 5th and 6th. On the 4th and 8th, 9th, and 10th days the death rates were the same, being about one-third of those for the 5th and 6th and one-half of those for the 7th day. Death seldom occurred on the 2nd or 3rd day or after the 11th day of disease.

TABLE II.  
*Fatal Cases.*

Day of disease on which death occurred.	No. of cases.
2nd	1
3rd	1
4th	5
5th	16
6th	17
7th	9
8th	5
9th	5
10th	4
11th	1
14th	1
16th	1
Total.....	66
Total No. of cases.....	140
No. recovered.....	74
" died.....	66 (47.1 per cent).

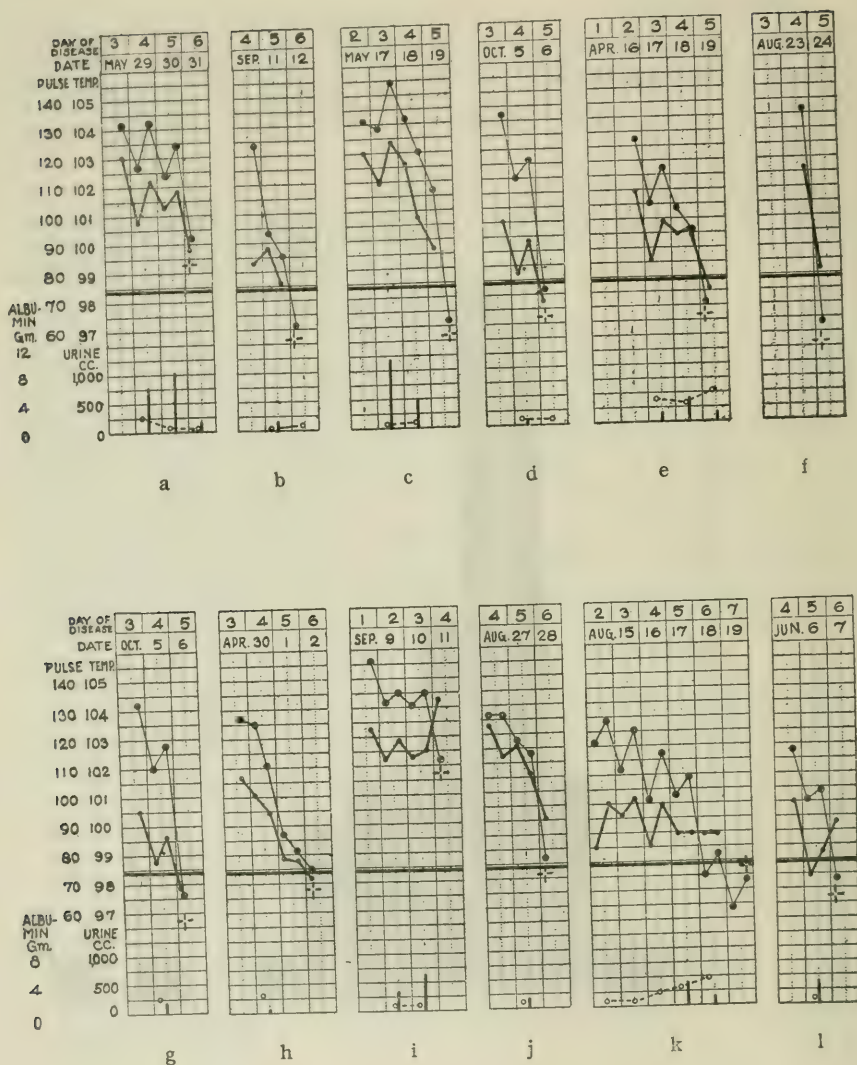
The temperature curves of these fatal cases (Text-fig. 5, *a* to *l*) are noteworthy as showing the rather rapid fall of temperature towards death. The initial fever in these cases was very high when observed during the early stage of the disease (above  $40^{\circ}\text{C}.$ ), and some showed



TEXT-FIG. 4. The chart shows the day of disease on which death occurred in 66 fatal cases.

nearly  $40^{\circ}\text{C}.$  even on the 4th or 5th day. Death took place with a temperature as high as  $39^{\circ}\text{C}.$  in some and as low as  $36^{\circ}\text{C}.$  in others. In a few instances the temperature fell to about  $37^{\circ}\text{C}.$  or even  $36^{\circ}$  or  $35^{\circ}\text{C}.$  on the day of death but registered a sudden rise to  $38^{\circ}\text{C}.$ , and in one case to  $40.6^{\circ}\text{C}.$ , just before death.





TEXT-FIG. 5, *a* to *l*. Fatal cases of yellow fever. (*a*) Case 40. Age 28 years. (*b*) Case 41. Age 24 years. (*c*) Case 42. Age 20 years. (*d*) Case 43. Age 19 years. (*e*) Case 44. Age 28 years. (*f*) Case 45. Age 25 years. (*g*) Case 46. Age 18 years. (*h*) Case 47. Age 18 years. (*i*) Case 48. Age 13 years. (*j*) Case 49. Age 11 years. (*k*) Case 10. Age 26 years. (*l*) Case 50. Age 24 years.

The analysis of the febrile reaction of man to yellow fever infection becomes important in a discussion of the febrile reaction of certain experimental animals when inoculated with the blood or organ emulsions from yellow fever patients.

*Disturbances in the Renal Functions.*—In almost all yellow fever cases the urine contains from the 1st day of illness more or less albumin which continues and increases in quantity. On the 2nd or 3rd day granular and epithelial casts appear, being abundant in severe cases. In fatal cases the quantity of urine rapidly decreases the 1st and following days until there may be complete suppression, perhaps anuria, for 24 hours or longer previous to death. The amount seldom exceeds 500 cc. In the non-fatal cases there is a similar diminution of urine during the first 4 or 5 days, but from that time there is a gradual return to the normal output. In many cases the quantity reaches 1,000 cc. on the 8th, 9th, 10th, or 11th day, while in some the secretion may be only 500 cc. or less for many more days. The casts and albumin are less in mild cases and disappear earlier than in the severe cases, in which they may persist for from 2 to 3 weeks. They are present in largest quantity some time during the 2nd week, and as early in rare instances as the 5th or 6th day. The average maximum of albumin is about 5 gm. per liter. In a few instances it went up to 18 gm. In fatal cases the amount of albumin was not very large, being about 1 to 2 gm. per liter, although there were a few instances in which it was somewhat over 5 gm. per liter during 24 hours. In a few apparently severe cases the amount of albumin was comparatively small, 1 to 2 gm.

During the 1st and 2nd weeks of the disease erythrocytes are always found in varying quantities in the urine.

*Jaundice.*—This is one of the most constant manifestations of yellow fever. The intensity of jaundice is, in the majority of cases, in proportion to the severity of the disease. In a very mild case it appears later and disappears sooner than in more severe cases and may be so slight that careful attention alone will reveal its presence. In average non-fatal cases jaundice may be detected on the 3rd day of the disease, first in the scleras and then in the lighter parts of the skin. On the 4th or 5th day the jaundice deepens, and the entire body assumes a light saffron to ocher-yellow hue. In milder cases

the height of jaundice is reached within the 1st week, while in severer cases the color goes on deepening for another week until the appearance of the patient varies from a grayish green to bright yellow. In fatal cases the jaundice may be pronounced or rather slight according to the period of the disease at which death occurred. The longer the patient lives the deeper is the jaundice as a rule. Jaundice becomes more evident after death, owing to the cessation of the blood circulation.

Bile pigments are present in the blood, pericardial, pleural, peritoneal, and cerebrospinal fluid, the adipose tissues, urine, various organs, muscles, glands, the skin, and mucous and serous membranes. The cartilages are also stained. The brains are not noticeably yellow. The urine becomes brownish yellow on the 2nd or 3rd day, and the color becomes deeper as the disease advances until it reaches the color of dark greenish brown or deep brownish yellow. In the bladder of patients dying during the 1st week the urine is of brownish yellow color.

Bile pigments are readily recognizable in the serum or plasma drawn after the 3rd day and give them a deep yellowish brown color in a later stage of the disease.

*Leucocytes*.—As a rule there is a slight hyperleucocytosis on the 1st day of the disease, but the number of leucocytes soon returns to normal, and in a few days a marked leucopenia sets in. There were a few exceptions, however, in which marked hyperleucocytosis was maintained for several days. The differential count showed a high percentage of polymorphonuclear leucocytes.

*Hemorrhages, Vascular Injection, and Herpes*.—Hemorrhagic diathesis is the third cardinal symptom of yellow fever and is never absent from fully developed cases.

During life hemorrhages are manifest in various forms, such as epistaxis, hematemesis, melena, hematuria, gingival hemorrhages, subconjunctival ecchymoses, and in occasional cases subcutaneous ecchymoses and petechiæ. Epistaxis is frequent and often profuse and may be one of the early symptoms. Hematemesis begins usually in the 1st week and becomes severer in the 2nd week. The vomitus is at first yellowish brown, but soon a coffee-ground color to which the term "black vomit" in yellow fever owes its origin. It is



a mixture of mucus, blood clot, and food in a semidigested state. This symptom may be absent in fatal cases, in which the coffee-ground contents may be first found in the stomach at autopsy. In severe but non-fatal cases the black vomit may occur in the 2nd week. Melena is a concomitant of hematemesis and is usually present in all severe cases. Gingival hemorrhages from swollen gums occur in many cases and are sometimes alarmingly persistent and profuse in severe cases. Hematuria is frequently observed, and in fact the urine of yellow fever patients always contains varying quantities of the blood corpuscles when examined under the microscope. Subcutaneous petechial hemorrhages of various sizes, from that of a pin-head to that of a split pea, were observed in several cases (Figs. 1 and 2).

During the 1st week the superficial vascular system seems to be dilated, and the blood-shot appearance of the conjunctival capillaries of the eyes is one of the most constant symptoms of the disease. The patients usually are flushed in the face in the early stage.

Herpes labialis is frequently present during the 1st week of the illness (Fig. 3).

*Pulse.*—Relative brachycardia is another well known characteristic symptom of yellow fever and has been observed in the majority of cases in Guayaquil. In many fatal cases, however, the pulse curve went up above that of the temperature a day or two before death. This disproportion of temperature and pulse is shown in the charts elsewhere recorded in this paper. A patient with a temperature of 39.5°C. may have a pulse of 80, and during the convalescent stage as slow as 45 or 50 beats per minute.

*Nausea and Vomiting.*—Nausea, accompanied by anorexia, is noted from the beginning and is soon followed by vomiting. Vomiting may occur in the beginning, however, or may begin several days later. The character of the black vomitus has already been described. It often appears bilious at first.

*Pains.*—Intense headaches, frontal, orbital, or general, are complained of by all patients during the first 3 or 4 days. Pains in the muscles of the trunk, loins, and calves, and sometimes in the arms and thighs are observed in all cases, being extremely intense in some. They are most marked during the first 3 days; later the patient may



make no reference to them unless questioned, being preoccupied probably by the cephalalgia. Epigastralgia is usually present and is almost intolerable in some severe cases. In mild cases the epigastric region is tender to pressure. Pains in the back in severe cases may be due to the acute nephritis. The liver is palpable, enlarged, and tender to pressure. The spleen is normal. The tongue is coated, with free red tip and edge. The lungs often show slight bronchitis.

*Onset and Course of Yellow Fever in Man.*

In the foregoing paragraphs the clinical features which constitute the disease known as yellow fever have been set forth. By bringing together the symptoms which occur concomitantly during the course of the infection, the reader can picture to himself the appearance of a mild, moderate, severe, or fatal case. A brief resumé is given here, however.

*Mild Infection.*—Onset with severe headache, coated tongue with red tip and edge, suffusion of conjunctivæ, myalgia, anorexia, and nausea, accompanied by a temperature of about 39° or 39.5°C., usually without chill. The patient is rather ill, but still able to go about. There is a moderate hyperleucocytosis which presently may drop to normal or to leucopenia. The pulse is relatively slow. A trace of albuminuria and icterus are present during the next few days. All symptoms rapidly disappear within a week or a little more.

*Moderate Infection.*—All the symptoms just described are present, together with black vomit during the 1st week. The patient is ill enough on the 1st day to go to bed. The presence of albumin and casts in the urine, oliguria, and icterus are rather pronounced and may persist for 10 days or more, when convalescence begins. Gastralgia is present. The liver is palpable and tender.

*Severe Infection.*—All symptoms are much aggravated, and there may be rigor and a fever around 40°C. Epistaxis, hematemesis, hematuria, melena, and gingival hemorrhages follow. Icterus is intense, and the urine diminishes rapidly as the disease advances, and great quantities of albumin, casts, and bile pigments are contained in it. The patient may become delirious at the end of the

1st week. The epigastric pain is severe. Profuse hemorrhages, hypothermia, and exhaustion ensue. By the 12th day the urine begins to increase in volume and continues to do so for subsequent days. With the increased urine the patient begins to improve, and within another week or two all the symptoms except jaundice and albuminuria gradually disappear.

Parotitis and a secondary fever on the 14th to 16th day were observed in certain cases as complications.

*Fatal Infection.*—The onset of the yellow fever that ends in death is the same as that observed in the severe non-fatal cases. Both are very grave from the beginning. Some cases appear to be in a state of exaltation, with brightly flushed face and blood-shot eyes, when brought in on the 2nd day, but the bloody vomit soon begins, and the patient is rapidly seized by an agony of pains. Albumin, casts, and blood cells are present in the urine. On the 3rd or 4th day there may be total anuria. The patient soon becomes delirious, then comatose, and dies in convulsions. There may be a sudden drop in temperature before death or in some instances a sudden rise to near 40°C. When death occurs after the 6th day the temperature is already low, sometimes below 37°C. Jaundice is always present in fatal cases. In some cases the black vomit, or rather hematemesis (still quite bright red) occurs near death or may be found at autopsy.

The foregoing summary is intended only to give a very general impression, the details being recorded in other papers.

#### *Autopsy Findings in Yellow Fever in Man.*

This part of the subject is important in completing our knowledge of the disease as it affects man. All the clinical manifestations are only the apparent characteristics of the disease and are explained and extended by the pathological findings.

Postmortem rigor and lividity are pronounced. There is intense jaundice throughout the entire body. The nostrils and mouth may be partly filled with blood clots and the face smeared with blood. The region about the anus is often stained with melena. Uterine hemorrhages were observed in young women. The skin, subcutaneous tissues, and muscles are yellow.

*Lungs.*—Often edematous; show marked hypostasis; crepitant. In every case there were ecchymotic hemorrhages, variable in size and distribution. The size varies from a few millimeters square to the size of a split pea, and is sometimes as large as a pigeon's egg. The hemorrhagic foci are sharply defined in some and diffuse in others. If recent they are vivid red, if several days old bluish red. On section they are seen to extend deep into the substance of the lungs, some into the interior. They are discrete and multiple, and the number varies in different cases and in the two lungs.

*Heart.*—Often shows hemorrhagic foci in the pericardium, which contains icteric fluid and is often dilated on the right side and in diastolic condition. The myocardium is grayish yellow, brittle, and cloudy. Numerous punctiform ecchymoses are frequently found on the surface. The endocardium is clear, with occasional punctiform hemorrhages along the papillary muscles. The valves are intact.

*Liver.*—Hyperemic and often somewhat enlarged. The color varies, yellowish brownish red, ocher-yellow, saffron-yellow, light greenish yellow, brownish yellow. The whole may be of uniform color or shaded, blended, or minutely mottled. The yellower or greener color indicates a more advanced stage of degeneration, in which the parenchyma is brittle and tears easily when handled with forceps. In a brownish red liver the tissue still retains much of its normal consistency. Minute ecchymotic spots are sometimes visible on the surface.

*Gall Bladder.*—Usually full of deep greenish yellow bile. There may be multiple ecchymoses in the wall.

*Kidneys.*—Enlarged, highly hyperemic, and reddish yellow in color. Varying number of ecchymoses, some punctiform and some as large as a bean, may be present in the capsule, which strips easily. In a few instances punctiform hemorrhages were found in the cortex. On section hyperemia is noticed along the junction of the cortex and medullary portion. The cortex is broader and shows general swelling and cloudiness. The medulla is succulent and icteric. The renal pelvis may be free, but not infrequently it contains a blood clot, or numerous punctiform hemorrhages are seen irregularly situated. The kidneys, therefore, show acute parenchymatous inflammation.



*Suprarenal Glands.*—Appear to be congested and more friable than normally. In one instance an extensive hemorrhage was found in the perinephritic adipose tissue.

*Gastrointestinal System.*—The stomach usually contains a viscid semifluid of coffee-ground appearance, known as the black vomit or *vomito negro*. In some instances freshly extravasated blood gives the gastric contents a dark reddish hue, or they may be dark green in color with black particles. The serosa is free from any ecchymoses, but the mucosa is intensely injected with more or less numerous ecchymoses, particularly near the cardia.<sup>3</sup> The small intestines as well as the colon are similarly affected and contain a tarry fluid (melena).

*Bladder.*—Except for occasional ecchymoses on the serosa nothing special has been observed. The bladder is often full.

*Uterus and Ovaries.*—Intense injection of the endometrium and sometimes hemorrhage in the uterus. The ovaries are somewhat injected. In one instance there was hemorrhage of both ovaries.

*Testicles.*—No change found.

*Spleen.*—Apparently normal.

*Lymphatic Glands.*—Swelling and hemorrhages were observed in the bronchial, mesenteric, and other lymphatic glands. This condition, as well as the constant ecchymoses in the lungs, seems to have received little attention from previous investigators.

*Serous Membranes.*—Pleuræ, peritoneum, and omentum are usually free from hemorrhages.

*Skin and Mucous Membranes.*—Occasional petechial hemorrhages were observed.

*Nervous System.*—The central nervous system is macroscopically unchanged. The cerebrospinal fluid is icteric and the brain edematous. The membranes are injected.

### *Histological Findings.*

*Lungs (Figs. 4 to 7).*—In the majority of instances areas of hemorrhage of varying extent are found and also small foci of infiltration with polymorphonuclear leucocytes. The alveoli are filled with

<sup>3</sup> This has been constantly observed by Dr. Pareja in his long experience.



polymorphonuclear and large endothelial cells and red blood corpuscles. The thrombi in some vessels are similar to those seen in infarctions.

*Liver (Figs. 8 to 11).*—The liver is necrotic in the large proportion of cases, and the parenchyma is full of extravasated blood. Only around the vessels are the living cells found, better preserved around the central vessel than around the portal canal. A moderate amount of pigment is sometimes found within the bile capillaries. The blood is distributed throughout the necrotic areas and not always confined to the blood vessels. Mitotic figures are met with among the liver cells. Most liver cells are vacuolated, and some are distended with vacuoles. Hemorrhagic areas are found. Numerous red-staining cells mingle with those less degenerated. There are a few granules in the liver cells but no pigment. In the region of the portal canal small foci of lymphoid and plasma cell infiltration are encountered.

*Kidneys (Figs. 12 to 15).*—The convoluted tubules are somewhat dilated with granular excretion. There are many deeply stained hyaline and granular casts. The epithelium of the convoluted tubules shows granular and somewhat vacuolated cytoplasm. The glomeruli are moderately or considerably injected; there is rather marked injection of the medulla with several hemorrhagic areas.

*Stomach.*—A few minute foci of infiltration with polymorphonuclear leucocytes; in certain areas a more diffuse infiltration of lymphoid and plasma cells (some bacilli). The superficial portion of the mucosa is markedly congested. The mucosa is somewhat injected in places.

*Large and Small Intestine.*—Injection and occasional hemorrhages.

*Heart.*—The muscle fibers show one or more vacuoles situated in the central portion, suggestive of fat. Certain fibers appear somewhat swollen. The nuclei are large and vesicular.

*Spleen.*—Numerous large phagocytes containing red cells. Follicles atrophied in one case; in another much blood in the pulp. A large number of phagocytic cells appear in certain areas. These contain red cells and red-staining granules.

*Lymph Nodes.*—(1) Some degeneration and phagocytosis centrally situated in the follicles. (2) Peripheral sinuses filled to a large extent

with polymorphonuclear leucocytes and a small number of phagocytic cells. The lymph follicles show small areas of degeneration centrally situated.

*Pancreas.*—Certain of the gland alveoli show small groups of degenerated cells. Sometimes there are no lesions.

*Adrenals.*—There is a marked degree of parenchymatous degeneration, affecting chiefly the medulla, and considerable congestion. In some cases the adrenals are intact.

*Nervous System.*—Nothing abnormal.

#### SUMMARY.

The clinical and pathological features of the yellow fever prevalent in Guayaquil conform with those described by other investigators of this disease as it has occurred elsewhere, both epidemically and endemically.

#### EXPLANATION OF PLATES.

##### PLATE 31.

FIG. 1. Patient Ch. The photograph, which was taken on the 5th day of the disease, shows large and small areas of subcutaneous hemorrhage on chest and arms, also on the temple. The blotch on the left breast was caused by the application by the patient of mustard plaster. The patient died on the 7th day.

FIG. 2. A patient who was severely ill but finally recovered. There were extensive subcutaneous hemorrhages all over the body of a mottled appearance but with no actual circumscribed spots.

FIG. 3. Patient Co. The photograph, which was taken on the 3rd day of the disease, shows herpes labialis, particularly of the lower lip. The patient died on the 4th day.

##### PLATE 32.

FIGS. 4 to 7. Sections of lung from four different cases of yellow fever, fixed with Zenker's fluid and stained with eosin and methylene blue. Hemorrhagic areas, varying in extent, can be seen in all these lesions. They are well defined with respect to the normal tissue (Fig. 4). There is a varying degree of edema. Fig. 4 is from a patient dying on the 6th day, Fig. 5 from a patient dying on the 5th day, Fig. 6 from a patient dying on the 8th day, and Fig. 7 from a patient dying on the 7th day.  $\times 150$

## PLATE 33.

FIGS. 8 to 11. Sections of liver from cases of yellow fever, fixed with Zenker's fluid and stained with eosin and methylene blue. The general character of the lesion consists in necrosis and vacuolization of the liver cells. Some groups of cells are completely disintegrated. The necrotic areas are occupied by debris and hemorrhage, particularly marked in Figs. 10 and 11. Fig. 8 is from a patient dying on the 6th day, Fig. 9 from a patient dying on the 5th day, Fig. 10 from a patient dying on the 8th day, and Fig. 11 from a patient dying on the 7th day.  $\times 150$ .

## PLATE 34.

FIGS. 12 to 15. Sections of kidney from cases of yellow fever, fixed with Zenker's fluid and stained with eosin and methylene blue. The general character of the lesion is the same in all these sections: swelling and degeneration of the renal epithelia in the tubules with varying degrees of hemorrhage into the connective tissue. The glomeruli are highly congested; some of the epithelia of the tubules are vacuolated and desquamated. The lumina of the tubules are filled with granular casts. Fig. 12 is from a patient dying on the 6th day, Fig. 13 from a patient dying on the 5th day, Fig. 14 from a patient dying on the 8th day, and Fig. 15 from a patient dying on the 7th day.  $\times 150$ .



FIG. 1.



FIG. 2.



FIG. 3.





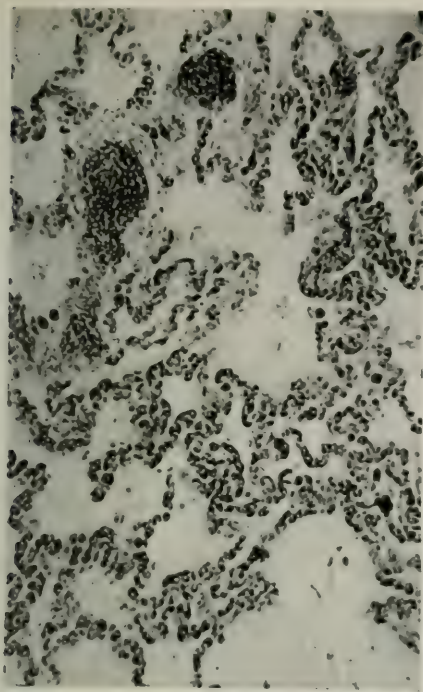


FIG. 4.

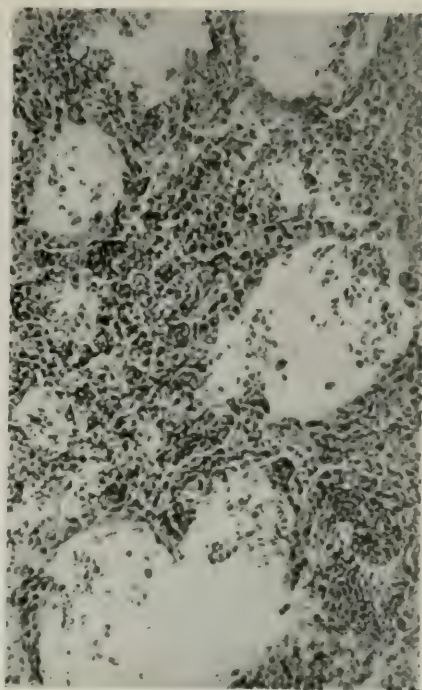


FIG. 6.

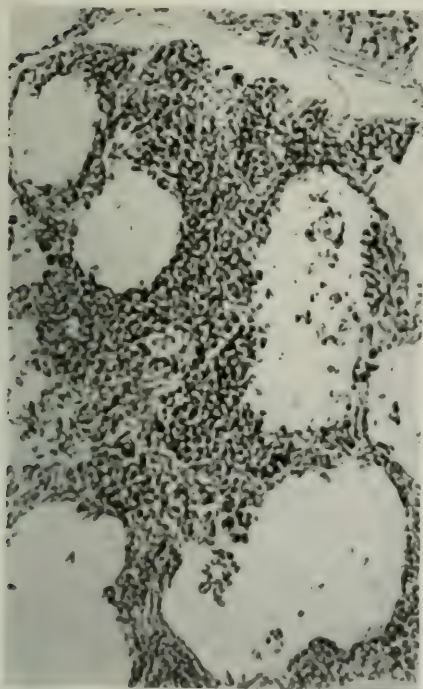


FIG. 5.

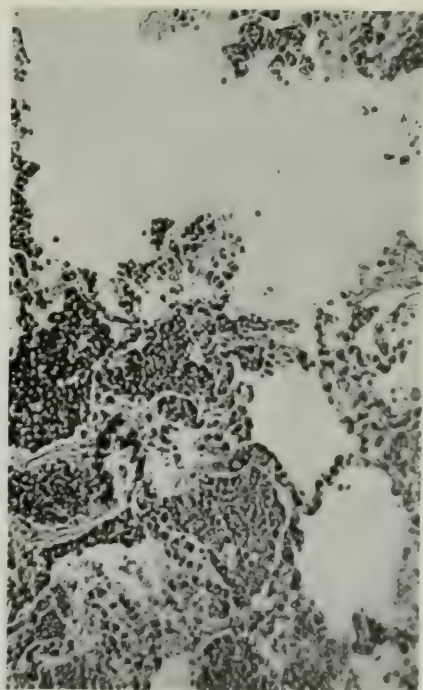


FIG. 7.

(Noguchi: Etiology of yellow fever. I.)



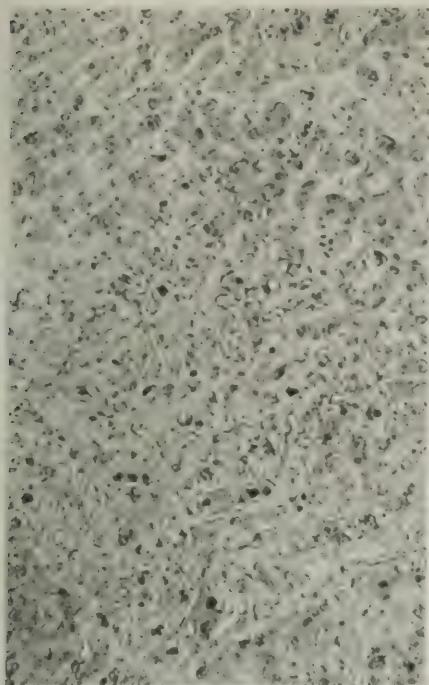


FIG. 8.

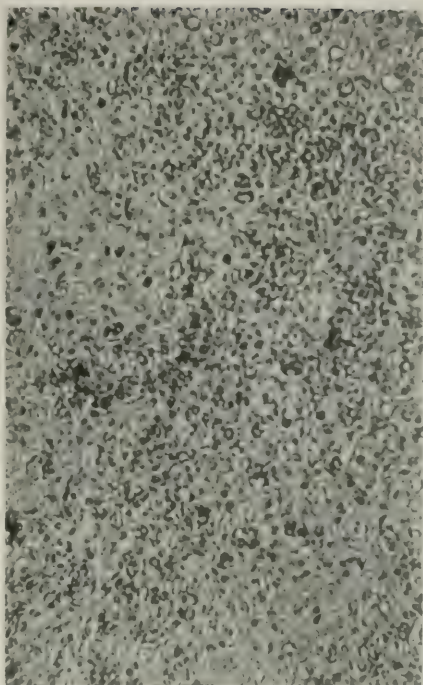


FIG. 10.

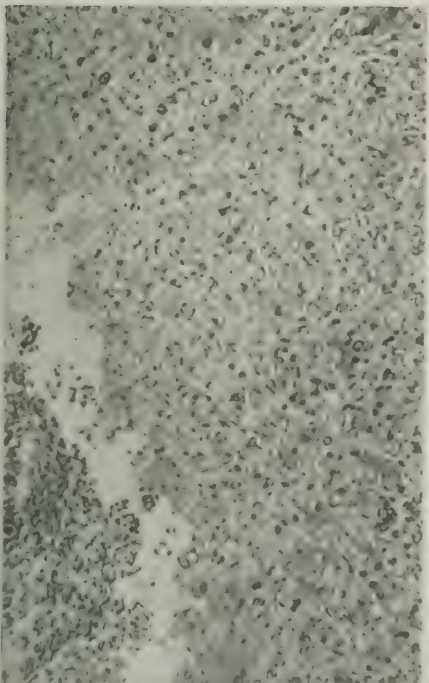


FIG. 9.

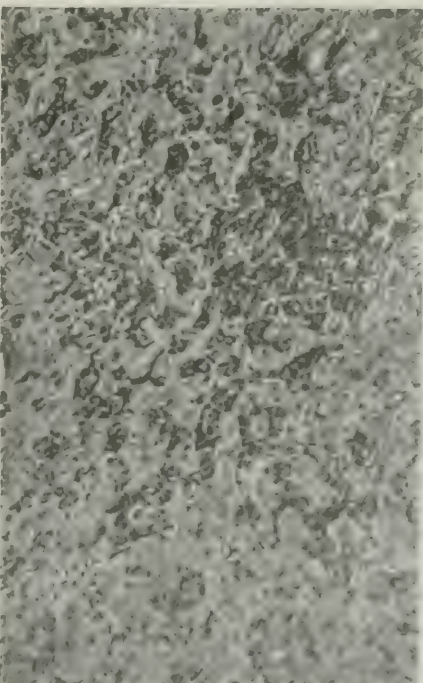


FIG. 11.

(Noguchi: Etiology of yellow fever. I.)





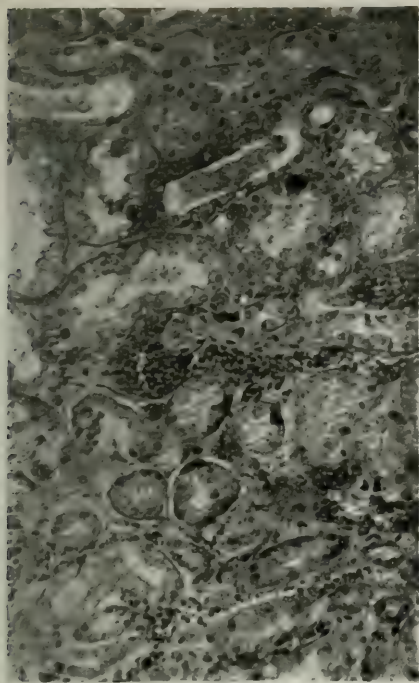


FIG. 12.

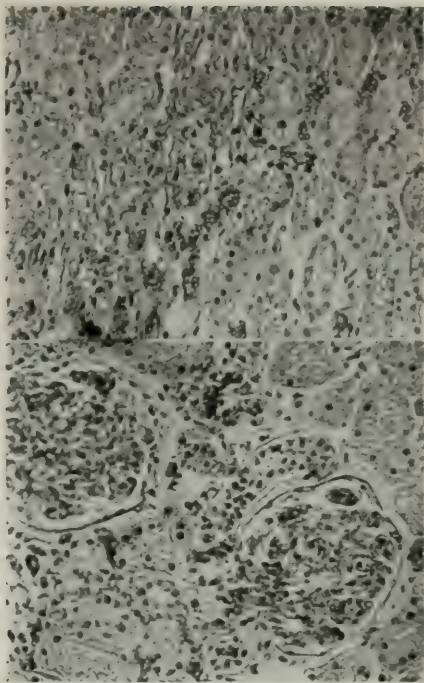


FIG. 14.

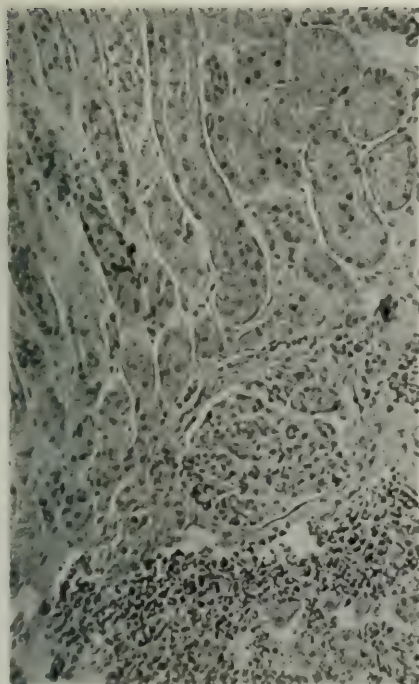


FIG. 13.

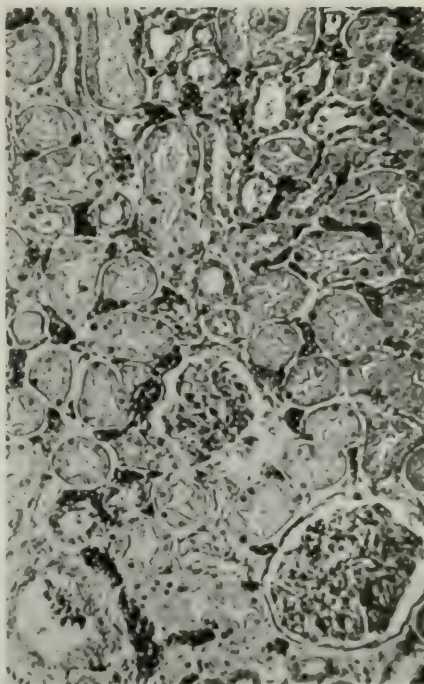


FIG. 15.

(Nozuchi; Etiology of yellow fever. I.)



## ETIOLOGY OF YELLOW FEVER.

### II. TRANSMISSION EXPERIMENTS ON YELLOW FEVER.

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PLATE 35.

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For the purpose of transmitting yellow fever to animals experiments were made to reproduce the disease in whatever kind of animal was available by inoculation with the blood from patients admitted to the Yellow Fever Hospital in Guayaquil during my stay there. The blood was drawn from the median basilic vein of the patient at various stages of the disease, mainly during the 1st week, with a sterile Luer syringe and injected before coagulation into the peritoneal cavity (in mammals) or into the pectoral muscles (in birds). 10 cc. of blood were usually taken and distributed among from one to four animals in each instance. When more than one kind of animal was used for one patient correspondingly larger quantities of blood had to be drawn. The following animals were employed: ringtail monkey, rabbit, guinea pig, cat, dog, donkey, guatusa,<sup>1</sup> comadreja,<sup>2</sup> and ozo-melero<sup>3</sup> among the mammals; pigeon, paloma de tierra,<sup>4</sup> bluebird, mantas,<sup>5</sup> blackbird, parrakeet,<sup>6</sup> reedbird, blancos,<sup>7</sup> and diostede<sup>8</sup> among the birds.

<sup>1</sup> *Dasyprocta aguti* (animal akin to rabbit).

<sup>2</sup> *Bassariscus bassaricyon* (?) (resembling an opossum).

<sup>3</sup> *Choloepus didactylus* (two-toed sloth).

<sup>4</sup> Family of Peristerinæ (ground-dove).

<sup>5</sup> Siskin (probably).

<sup>6</sup> Family of Bolborhynchus.

<sup>7</sup> Similar to a magpie, but white.

<sup>8</sup> Genus *Pteroglossus*.



All the animals inoculated were kept under observation at least 1 month before being discarded as negative, even when no symptoms were observed. An animal which showed a rise of temperature after a period of several days was closely watched, and when more than one of the animals of the series receiving the same blood became febrile one was killed for examination and further passage made in the same species of animal. As will be seen later (Case 2) such a step was essential with some specimens of yellow fever material, as by this means alone was it possible to reproduce an experimental condition resembling yellow fever on the third animal passage.

None of the birds showed any definite symptoms when first inoculated with the blood of yellow fever patients, but upon subsequent injection of the organ emulsion of a guinea pig with the experimental yellow fever<sup>9</sup> parrakeets, mantas, blackbirds, reedbirds, and paloma de tierras died within 24 to 72 hours. There were no lesions except for hyperemia of the visceral organs. The pigeons, bluebirds, and toucans showed no definite reaction. This rather rapid death following the second inoculation may have been due to an anaphylactic phenomenon. Rabbits and guatusas showed a temperature of 40–41°C. on the 4th to 5th day after inoculation but became normal after 48 hours. There were no other symptoms, and the animals remained well during the period of observation (1 month). The ringtail monkeys showed a similar but more prolonged fever reaction lasting several days. The animals appeared depressed after 48 hours and refused to take food. The conjunctivæ were injected for several days, but there was no jaundice. Unfortunately the number of experiments with this animal was limited to five because of the difficulty in securing them in sufficient numbers. Donkeys showed no reaction to the injection of yellow fever blood in large quantities.

Apparently none of the animals enumerated above possesses a sufficient degree of susceptibility to enable the yellow fever virus to multiply and reproduce the symptoms and lesions known as yellow fever in man. It is possible that some of them had an extremely mild or atypical form of the infection.

<sup>9</sup> For the sake of brevity the condition induced by inoculation will be termed "experimental yellow fever." Further evidence bearing on the validity of this term will be presented in successive papers of the series.

Since guinea pigs could be secured more easily, and in view of the success of Inada and Ido<sup>10</sup> with this species in the study of infectious jaundice, they were extensively used during the present study. Guinea pigs are native to Ecuador and are found wild in the mountainous regions.<sup>11</sup> All except 60 of the guinea pigs which were used in these experiments (which were taken from New York) were reared in the mountains and shipped to the Yellow Fever Hospital. They stand captivity well for several months.

### *Experiments with Guinea Pigs.*

*Blood and Organ Emulsions.*—Of 74 guinea pigs inoculated with the specimens of blood from 27 cases of yellow fever, 8, representing 6 cases, came down with the symptoms resembling human yellow fever. In one instance, however, the reproduction of the disease required three successive inoculations in guinea pigs. In this instance one of the animals showed a rise of temperature on the 5th day and was killed for examination and transfer on the 6th day. The liver was degenerated, and the kidneys were highly congested. A few hemorrhagic spots were found in the lungs. In a further passage of the organs to two new guinea pigs there was a similar febrile reaction with ecchymoses in the lungs but only a trace of icterus. On transfer of the second passage material typical hemorrhages, icterus, and albuminuria were induced. With the materials from only one of several autopsies was a positive transmission obtained. The details of the transmission experiments follow.

The first positive transmission was obtained in the case of Patient A.

*Case 1 (Text-Fig. 1, a).*—A. A., female, age 17 years; servant, native of Latacunga (9,055 feet above sea level). Had come to Guayaquil 3 months previously.

Onset, July 14, 1918. Headache, chills, fever, and severe pains throughout the body. July 15. Vomited chocolate-colored matter and felt pains in epi-

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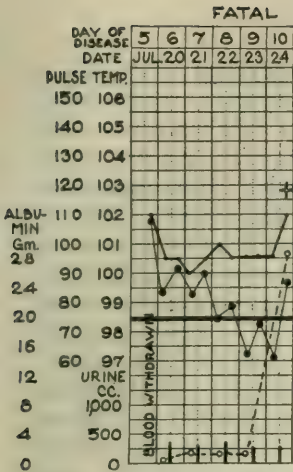
<sup>10</sup> Inada, R., Ido, Y., Hoki, R., Kaneko, R., and Ito, H., *J. Exp. Med.*, 1916, **xxiii**, 377.

<sup>11</sup> Guinea pigs are in the market for food in Ecuador, but the municipality of Guayaquil has forbidden their being kept in the city on account of the possibility of spreading bubonic plague.

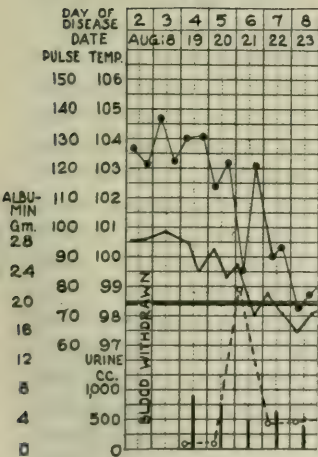
gastric region. July 19. Admitted to hospital. Was very sick and vomited dark bloody matter. Conjunctivæ intensely injected and distinctly jaundiced. Liver tender but not palpable. General jaundice of the skin. Blood drawn from median basilic vein into citrate solution and inoculated into guinea pigs immediately and also after 3 days cultivation at 25°C. July 20. Patient felt better, but gums began to bleed. Abdominal pains not marked. July 22. Condition worse. Patient very ill, vomited constantly ("coffee-grounds vomitus"). Jaundice very marked. Liver tender and swollen. Tumefaction of thighs because of menstruation. July 23. Delirium and insomnia with intense epigastric pains. July 24, 9 p.m. Died.

*Autopsy.*—Performed 11 hours post mortem. Well nourished young woman, with well marked rigor mortis and lividity. Body cyanotic and mottled. Pronounced general jaundice. Mouth and nostrils contained dark, partly dried blood clots, and face was badly smeared with dried blood. Similar blood specks were found on the hands and the genital regions, the skin of the thighs being extensively excoriated owing to hemorrhages from the uterus. No petechiæ or subcutaneous ecchymoses present. Lungs edematous. Pronounced hypostatic congestion of the dependent parts. Several ecchymoses of pea size present. Pleura free, containing moderate amount of deeply jaundiced clear fluid. On section the hemorrhagic spots of the lungs were seen to extend many centimeters into the parenchyma; some occult foci of similar ecchymoses in the interior. Some old and some new foci of tuberculosis in the upper lobes. Pericardium apparently free from any changes; scanty icteric fluid in the cavity. Heart normal in size; in diastolic state, containing fluid blood in right heart. Few minute ecchymoses on the surface. Muscles pale, flabby, and brittle. Endocardium and valves normal except for a few petechial hemorrhages on the papillary muscles. Liver pale ocher-yellow in color, fatty, and streaked with congested areas. Color on section uniformly yellow; parenchyma friable. Stomach distended with gas, containing blackish bloody mucous material; mucosa hyperemic, especially a few minute hemorrhagic spots near the cardia. Intestines filled with dark tarry contents; mucosa hyperemic. Kidneys swollen and hyperemic, capsules non-adherent; on section numerous ecchymoses in the pelvis; increase of the cortical layer and cloudy swelling. Deeply jaundiced. Suprarenal glands hyperemic. Spleen and pancreas apparently normal. Ovaries congested and uterus hyperemic, containing extravasated blood in the cavity. Small amount of deeply yellowish urine in bladder.

*Animal Inoculation.*—The blood, drawn on the 5th day of disease, was used in two different ways. One portion was immediately inoculated into the peritoneal cavity of two guinea pigs, and the other was put into a mixture of Ringer solution 3 parts and human serum 1 part with 0.3 per cent neutral agar, then well mixed and covered with a layer of paraffin oil. This semisolid mixture was placed in a thermostat at room temperature for 3 days (20–25°C.) and then injected into two guinea pigs intraperitoneally. The amounts of the original blood injected into each guinea pig were from 2 to 3 cc. The protocols follow.



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TEXT-FIG. 1, a to f. Cases (a) Case 1. Age 17 years. (b) Case 2. Age 20 years. (c) Guinea

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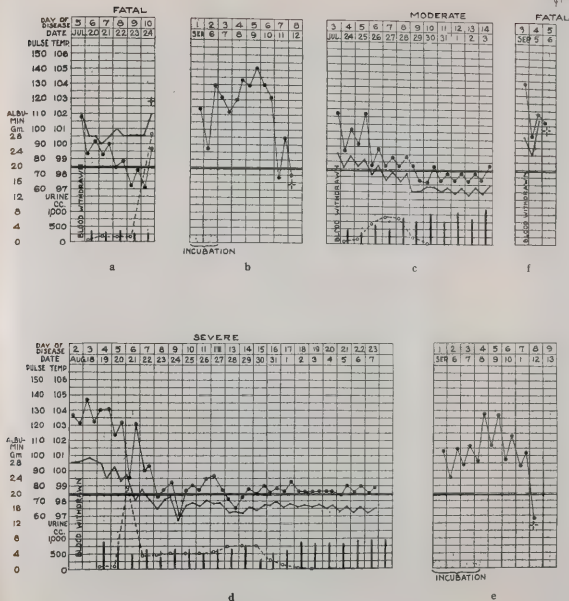
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TEXT-FIG. 1, a to f. Cases of yellow fever in which positive transmissions to guinea pigs were obtained. (a) Case 1. Age 17 years. (b) Guinea Pig 129 A. Strain, Case 1. (c) Case 2. Age 23 years. (d) Case 3. Age 20 years. (e) Guinea Pig 353 C. Strain, Case 3. (f) Case 4. Age 15 years.

*Guinea Pig 50.*—July 19, 1918. Inoculated with 3 cc. of the fresh blood intraperitoneally. The temperature rose to 39.6°C. on the 6th day, dropped to 38.5° on the 7th, again rose to 39.8° on the 8th, was 40° on the 9th, and 39.7° on the 10th day. The animal became less active, and on the 11th day it had a temperature of 39.1°, which continued for 3 days and then returned to normal (38–38.5°). On the 11th day the scleras appeared icteric, and the capillaries of the conjunctiva much suffused. The animal recovered completely in 15 days. On a subsequent injection (Aug. 10, 1918) with material taken from other guinea pigs inoculated with the enriched blood of the same patient this animal failed entirely to react.

*Guinea Pig 51.*—July 19, 1918. Inoculated intraperitoneally with 2 cc. of the same lot of blood. The temperature was 39.9°C. on the 9th day, 40.5° on the 10th, and 39.2° on the 11th, returning gradually to normal (38.5–39°). There was a suspicion of jaundice on the scleras on the 12th day which disappeared in a few days. This animal remained well and was subsequently (Aug. 10, 1918) tested against material taken from this case. It failed to react.

*Guinea Pig 80.*—July 22, 1918. 4 cc. of the mixture of the citrate blood and the culture medium, which had been incubated for 3 days, were injected intraperitoneally into the guinea pig. The temperature was 40°C. on the 5th day, 40.1° on the 6th, and 38° on the 7th, when the animal became distinctly jaundiced. Death occurred the same day (July 29).

*Autopsy.*—Marked general jaundice. No definite subcutaneous ecchymoses. Dried blood specks around the nostrils. Mucous membranes very yellow. Lungs congested; showed few disseminated ecchymotic spots which on section were seen to extend deeper into the parenchyma. The size varied from a minute point to that of a split pea, and the color from bright red to dark bluish red. The contour of these areas was usually sharply demarcated from the normal tissue. Lungs edematous and the lower lobes highly hypostatic. Pleuræ not affected. Right heart appeared dilated and contained semicoagulated cyanotic blood. A few punctiform hemorrhages on the anterior surface. Muscles friable. Liver yellowish brown in color and mottled with areas of congestion. The markings of the lobules were prominent. Consistency firmer and less succulent than the normal. Stomach distended with undigested food partly tinted with the dark blood which escaped from the hemorrhagic spots on the highly congested mucosa, especially marked near the cardia. Intestines highly hyperemic and showed numerous spots of hemorrhage along the mucosa. Contents bloody. Kidneys intensely hyperemic and more or less swollen, showing a few punctiform hemorrhages in the cortex. On section the pelvis was filled with blood and there were some ecchymoses. The cortex was broad and red, and the medulla succulent and cloudy. Adrenals hyperemic and swollen. Spleen, pancreas, bladder, and testes apparently unaffected. Along the abdominal wall the muscles were congested and showed some disseminated ecchymoses.

*Microscopic Examination.*—The blood was apparently free from any micro-organism when examined under the dark-field microscope. Emulsions were

prepared from the liver, kidney, and adrenals for examination by dark-field and also for further passage. A very few actively motile organisms belonging to the genus *Leptospira* were demonstrated in the liver and kidney emulsions but not in the adrenal.

*Passage.*—Two guinea pigs were immediately inoculated with a mixture of the emulsions of the liver and kidney from Guinea Pig 80 and a further generation of this strain was obtained in the two following guinea pigs.

*Guinea Pig 80 A.*—July 29, 1918. The organ emulsion (2 cc.) was inoculated intraperitoneally. The animal showed a temperature of 41.3°C. on the 4th day, 40.6° on the 5th, and 38.7° on the 6th, when the color of the skin and mucosa became intensely icteric. Death occurred at 3 p.m. on Aug. 3 while the animal was being placed in a mosquito cage with the stegomyias.

*Autopsy.*—The general appearance was the same as that of Guinea Pig 80. The urine contained bile pigment, albumin, and abundant casts. The leptospira could not be demonstrated either in the blood, liver, or kidney.

*Guinea Pig 80 B.*—July 29, 1918. The emulsion was applied to the scarified surface of the skin. The highest temperature (morning) was 39.9°C. on the 7th day. It went down to 38.8° on the 9th day, when the animal became very yellow. The animal died on the 10th day (Aug. 7).

*Autopsy.*—About the same as in the foregoing experiment except for a higher degree of degeneration of the liver, which was ocher-yellow, exactly as in some human cases. No leptospiras were demonstrated by the dark-field microscope, but a further successful transfer was made.

*Guinea Pig 81.*—July 22, 1918. 5 cc. of the enriched blood of Patient A. were injected intraperitoneally. The temperature was 40.3°C. on the 6th day, 40° on the 7th day, 39.9° on the 8th, and 38.7° on the 9th day, when jaundice became noticeable. The animal was found dead on July 31.

*Autopsy.*—Lesions similar to those already described in Guinea Pig 80. The stomach was filled with a semifluid blackish gray matter (digested blood). Dark-field examination of the blood, liver, and kidney failed to demonstrate any organism. Transfers were made to six guinea pigs. The protocols are given below.

*Passage.*—July 31, 1918. Six guinea pigs were inoculated with the emulsions of the kidney and liver, intraperitoneally and also percutaneously after depilation.

*Guinea Pig 124.*—July 31, 1918. Kidney emulsion (1 cc.) intraperitoneally. The animal had a temperature of 40.1°C. on the 4th, 40.5° on the 5th, 39.6° on the 6th, and 39.9° on the 7th day. It continued to show high temperature for 14 days, but jaundice was not present at any time. It was discarded after 30 days (Aug. 30).

*Guinea Pig 125.*—The same material was used as in the foregoing experiment. The animal had a temperature of 40°C. on the 4th and 40.1° on the 5th day. After that it was 39.5° or less until the 11th day, when the animal became quite icteric. It collapsed while being used for infecting stegomyias in a cage (Aug. 10,

4 p.m.), and was killed for examination of blood, liver, and kidney. Considerable numbers of leptospiras were found in the blood and organs. Further transfers were made with success. A culture was obtained from the heart's blood, but within a week a fungus contamination destroyed it.

*Passage. Guinea Pig 126.*—July 31, 1918. The liver emulsion (1 cc.) of Guinea Pig 81 was inoculated into the peritoneal cavity. The temperature went up to 40°C. on the 5th, 6th, and 9th days; the animal showed definite jaundice on the 10th day and died on the 11th. The chief symptoms were all very marked.

*Guinea Pig 127.*—This was a duplicate of the foregoing experiment. The animal died of a secondary infection within 3 days.

*Guinea Pig 128.*—Percutaneous inoculation with a mixture of the liver and kidney emulsions of Guinea Pig 81. The animal had a temperature of 40°C. during the 10th, 11th, and 12th days, but it had normally registered as high as 39.5°. There was a trace of jaundice on the 13th day, which soon disappeared. The animal remained well for 31 days and was discarded as negative.

*Guinea Pig 129.*—This was a duplicate of the foregoing experiment. Temperature 40.3°C. on the 7th and 40.1° on the 8th day, when a suspicion of jaundice appeared. It was killed on the 8th day in order to ascertain the lesions and obtain material for culture and transfers.

*Autopsy.*—Jaundice too slight to be definitely recognized. The lungs, however, showed the beginning of ecchymotic spots, and the liver and kidneys were highly congested. Dark-field examination failed to reveal any organism. Transfers were made to two guinea pigs on the same day (Aug. 7). One of them died with mild but typical lesions, while the other completely recovered after having shown a temperature of 40° and 40.4°C. on the 7th and 8th days.

Text-fig. 1, *b* is the chart of Guinea Pig 129 A, which succumbed to the intra-peritoneal inoculation of 0.5 cc. of a culture of this strain (Aug. 27–Sept. 5, 1918), with typical symptoms and lesions. The leptospira was demonstrated in the liver.

The foregoing experiments are of great interest as they indicate that the microorganism which was responsible for the disease and subsequent death of this patient was apparently successfully transmitted to the guinea pig. The failure of the unmodified blood to reproduce the disease in guinea pigs as contrasted with successful transmission by means of the same blood after a temporary enriching of the virus *in vitro* may be explained by the fact (as subsequent experiments concerning the properties of the serum of yellow fever patients and the properties of the organism indicate) that the inoculation of the fresh blood carried with it a certain amount of antibodies antagonistic to the development in the body of the guinea pig, while this



property is certain to be considerably modified when incubated in the culture medium employed. Moreover, as the organism is capable of multiplying outside the body at room temperature there is temporary enrichment of the organisms before inoculation into the guinea pig.

It is also shown in this experiment that the microorganism is variable in virulence, being able to produce a fatal infection in some, an abortive infection in others, and in still others no infection at all. The organism is capable of entering the body of the guinea pig in some instances by the smearing of the infective material. That there is an abortive form of the infection is shown by the immunity that it confers upon the guinea pigs which survived such an infection. The infective agent may be passed from one guinea pig to another by timely transmission.

A leptospira has been found to be associated with this case, but its demonstration by means of the dark-field microscope is not always successful; it may or may not appear in a subsequent passage. That this organism, notwithstanding the difficulty of demonstrating it, is etiologically related to the disease known as yellow fever in Guayaquil is made highly probable by subsequent experiments.

Fig. 1 shows the organism in the blood of a guinea pig inoculated with the culture of this strain.

The second positive transmission was obtained in the case of Patient P.

*Case 2 (Text-Fig. 1, c).—C. P., male, age 23 years; tailor, robust, a native of Cuenca (highland). Had been in Guayaquil a month.*

Onset July 20, 1918. Felt fever without chills; severe headache, photophobia, muscular pains throughout body, cramps in legs. No nausea. July 23. Admitted to hospital. Marked injection of the conjunctiva; slight jaundice; much albumin in the urine. Liver small but tender (Dr. Elliott). Temperature 38.9°C. Blood was drawn from the median basilic vein and injected into two guinea pigs (see protocols). July 24. Epistaxis in the morning. July 25. Nauseated easily; diarrhea. Little urine since yesterday. July 26. Jaundice advanced. Albuminuria + + +. July 31. Improving. Temperature normal. Aug. 2. Trace of jaundice. No albumin in the urine. Aug. 3. Discharged.

*Animal Inoculation. Guinea Pig 83.—July 23, 1918. Intraperitoneal injection of 4 cc. of the blood of Patient P. (4th day of disease). Temperature rose to 39°C. on the 4th and 40° on the 5th day. The animal was killed on the 6th day for further passages into two guinea pigs.*

*Autopsy.*—Lungs showed a few hemorrhagic spots. Liver congested and somewhat yellowish (fatty). Kidneys congested and cloudy, showing swelling on section. Gastrointestinal system hyperemic but showing no hemorrhages. Spleen normal.

*First Passage. Guinea Pig 88 A.*—July 28, 1918. Received 1 cc. of blood of Guinea Pig 88 intraperitoneally. The temperature rose to 39.9°C. on the 7th day; killed on the 10th day for examination and transfer.

*Autopsy.*—Slight icterus. The lungs were spotted with a few ecchymoses. Liver congested and perhaps fatty. Kidneys congested and showed cloudy swelling on section. Intestine hyperemic and showed scattered hemorrhagic foci.

The liver and kidneys were emulsified and used for inoculating two normal guinea pigs (see second passage). *Leptospiras* not found.

*Guinea Pig 88 B.*—Duplicate of No. 88 A. This animal showed a slight rise of temperature in 4 days, but during observation for further evolution of the reaction it became normal and was discarded on Aug. 22.

*Second Passage. Guinea Pig 89 A.*—Aug. 6, 1918. 1 cc. of the mixed emulsion of the liver and kidney of Guinea Pig 88 A was given intraperitoneally. The temperature went up to 40.9°C. on the 3rd, 39.6° on the 4th, and 40° on the 5th day, but fell to 38.6° on the 6th day. Epistaxis and icterus. The animal was found dead on the 7th day.

*Autopsy.*—Extremely jaundiced. Lungs hemorrhagic; liver yellowish; kidneys congested, with some ecchymoses. Stomach and intestines contained blackish matter and numerous hemorrhagic areas were present on the mucosa. *Leptospiras* were demonstrated in the liver and kidney but not in the blood. Transfers to two normal guinea pigs were made, but this strain was lost because of intercurrent infection.

*Guinea Pig 89 B.*—Duplicate of No. 89 A. This animal showed less fever than usual in the 1st week but 40°C. on the 10th day. It was kept under observation until Aug. 22, but nothing further developed.

*Guinea Pig 89.*—This was one of the two guinea pigs inoculated with the blood of Patient P. on July 23. This animal showed a temperature of 39.3°C. on the 4th, 39.9° on the 5th, 39.2° on the 6th, 38.9° on the 7th, 39.4° on the 8th, and 39° on the 9th day. It was found dead on the 10th day.

*Autopsy.*—Congestion of the lungs, liver, and kidneys, but no icterus. No transfer was made.

The third positive transmission was obtained with the blood of Patient G.

*Case 3 (Text-Fig. 1, d).*—M. G., male, age 20 years; servant, robust, a native of Ambato (8,435 feet above sea level); had been in Guayaquil 2 months.

Onset Aug. 16, 1918, 10 a.m. General pain and aching; headache; fever without rigor; no nausea, vomiting, or bleeding. Aug. 17. Admitted to hospital. Eyes injected, face flushed; much prostrated. Pains in the head and limbs were

intense. Albuminuria slight; no casts. Temperature 39.9°C. Blood taken for transmission experiments. Aug. 18. Albuminuria increasing; temperature 40.3°. Condition worse. Total leucocytes 9,200 (Dr. Elliott). Aug. 19. Albuminuria increasing and numerous casts in the urine. Temperature still 40°C. Aug. 21. Delirious; otherwise condition the same. Aug. 22. No urine obtained; gums bleeding; jaundice definite; remission in fever. Aug. 23. Decided improvement; temperature going down. Aug. 26. Beginning hematuria; increased icterus. Aug. 29. Continued hematuria; great prostration. Aug. 30. Hematuria diminished; general improvement. Sept. 1. Continued improvement; cessation of hematuria. Sept. 2. Continued improvement; intense jaundice. Sept. 3. Slight pain in bladder. Sept. 7. Discharged.

*Animal Inoculation. Guinea Pig 351.*—Aug. 17, 1918. 3 cc. of the blood of Patient G. (2nd day of disease) were given intraperitoneally. The animal was kept under observation until Sept. 7 but remained without any noticeable reaction except that its temperature rose to 40.1°C. on the 18th day. Discarded as negative.

*Guinea Pig 352.*—Aug. 17, 1918. 5 cc. of the same specimen of blood were given. Temperature 40°C. on the 7th day, 39.8° on the 8th, and 39.6° on the 9th, but later returned to normal (38.6° for this animal). Tested for immunity on Sept. 7 against the A. strain and found to be resistant.

*Guinea Pig 353.*—Aug. 17, 1918. 6 cc. of the blood from Patient G. were inoculated intraperitoneally. The temperature rose to 40°C. on the 7th and 39.6° on the 8th day, but dropped to 38° on the 9th day. The animal was markedly icteric and was killed for examination of the blood and organs as well as for transfer and cultivation of the virus.

*Autopsy.*—The lungs showed a moderate number of ecchymoses. Liver light yellowish brown in color. Kidneys showed acute parenchymatous nephritis; no ecchymoses. Stomach contained a few hemorrhagic foci near the cardia with blood-stained contents. Intestines hyperemic but with few hemorrhages. Intense jaundice everywhere. Spleen normal in appearance. After a long search a few leptospiras were discovered, but the number was so extremely small that preliminary examination revealed none. Transfers were made successfully to two normal guinea pigs, and cultures were also obtained. The organism was demonstrated in the blood of guinea pigs inoculated in later experiments with this strain (Fig. 2).

*First Passage. Guinea Pig 353 A.*—Aug. 26, 1918. Inoculated with 1 cc. of the blood of Guinea Pig 353. Except for a slight rise in temperature nothing happened during the period of observation (up to Sept. 10).

*Guinea Pig 353 B.*—Duplicate of the foregoing experiment. This animal came down with typical symptoms and lesions on Sept. 5, 1918; that is, on the 10th day after the transfer.

*Second Passage. Guinea Pig 353 C.*—Sept. 5, 1918. Inoculated with the emulsion of liver of Guinea Pig 353 B. The course of the infection was similar to that of the positive instance, with the temperature as shown in Text-fig. 1, *e*.



The fourth positive transmission was obtained with the blood of Patient Co. Successful transmission was also made with the liver emulsion of this patient, as described below.

*Case 4 (Text-Fig. 1, f).*—J. Co., male, age 15 years; servant, native of Cuenca (highland). Had been in Guayaquil a month and a half.

Onset Sept. 2, 1918. Headache and pains in waist and legs; fever. Sept. 4. Admitted to hospital. Face flushed; conjunctiva injected and slightly jaundiced; gums reddish and swollen; tongue coated, with red tip and edge. Herpes labialis present.<sup>12</sup> No epigastric pain. Blood drawn for inoculation. Sept. 5. Passed no urine, but appeared calm. Increased jaundice. In the afternoon became excitable, anxious, complained occasionally of dyspnea. Melena. Sept. 6. Coma began previous night. Black vomit. Complete anuria. Death occurred at 9 a.m.

*Autopsy.*—A partial autopsy within 1 hour after death. Liver highly degenerated and yellow. In the emulsion a few leptospiras were found. Kidney congested, cloudy swelling. No leptospiras.

*Animal Inoculation. Guinea Pig 435.*—Sept. 4, 1918. Intraperitoneal injection of 3 cc. of the blood of Patient Co. (3rd day of disease). The animal showed a temperature of 39°C. on the 8th, 40° on the 9th, 39.9° on the 10th, and 39° on the 11th day, but subsequently a normal temperature. It was discarded as negative after 25 days.

*Guinea Pig 436.*—Duplicate of the foregoing experiment. 5 cc. of the blood injected. The temperature rose to 39.9°C. on the 9th and 40.1° on the 10th day, coming down to 38.2° on the 12th day. The animal showed distinct icterus on the 11th and 12th days and died during the night of the latter.

*Autopsy.*—General jaundice. Hemorrhages in the lungs and gastrointestinal tract. Liver brownish yellow. Kidney highly congested; showed cloudy swelling on section. Other organs normal in appearance. Leptospira found under the dark-field microscope and in stained slides (Fig. 3).

The fifth positive transmission was obtained with the emulsion of the liver of the same patient, who died on the 5th day. The material was taken from the body within 1 hour, and the emulsion made with Ringer's solution was inoculated intraperitoneally into four guinea pigs, the quantity for each being 1 cc. A few distorted immobile leptospiras were demonstrated in this emulsion under the dark-field microscope.

*Guinea Pigs 444 and 446.*—Sept. 6, 1918. Received 1 cc. of the liver emulsion from Patient Co. Died on the night of the following day.

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<sup>12</sup> See Fig. 3, Paper I.



*Guinea Pig 447.*—Duplicate of the foregoing experiment. This animal never showed a reaction and died from an intercurrent infection after 22 days.

*Guinea Pig 445.*—Duplicate of the foregoing experiment. The animal had a temperature slightly above 39°C. for 6 days which rose to 39.9° on Sept. 13 and to 39.7° on the following day. Sept. 15. The animal was distinctly icteric and was killed for examination and transfer.

*Autopsy.*—Typical lesions in the lungs, stomach, liver, and kidneys. The blood, as well as the liver and kidney, showed a few leptospiras.

*Passage.*—From this animal the strain has been successfully passed through many generations. A culture also was obtained from a later passage.

The sixth positive transmission was obtained with the blood of Patient Ch., drawn from the median basilic vein on the 5th day of the disease and 48 hours previous to his death. In this instance 2 cc. of the fresh blood were directly inoculated into the peritoneal cavity of each of the two guinea pigs.

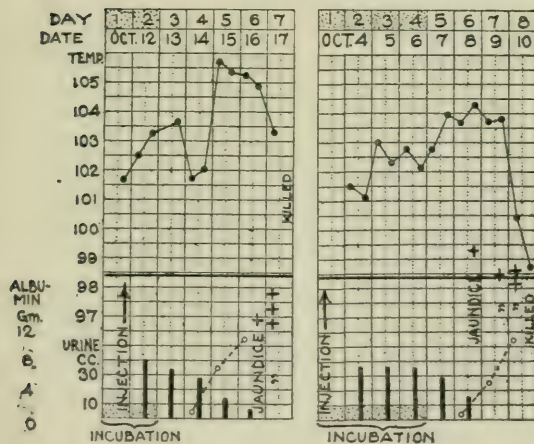
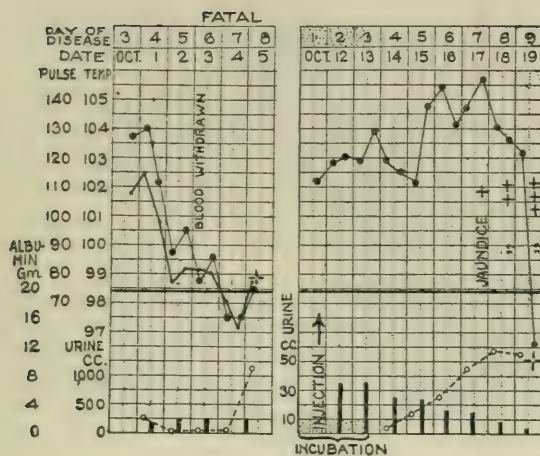
*Case 5 (Text-Fig. 2, a).*—E. Ch., male, age 30 years; well built man, a native of Guamote (highland). Had lived in Guayaquil for 8 months.

Onset Sept. 28, 1918. Sudden fever with chills, headache, pains in the limbs and back. Admitted to hospital on Sept. 30. Still complained of pains in the body and headache, with injection of the conjunctivæ; tongue coated, with free edge and tip; gums swollen. Albumin and casts in the urine, which was diminished in volume. Oct. 1. Scleras and skin somewhat yellowish. Oct. 2. All symptoms aggravated. Oct. 3. Chest showed several pea-sized hemorrhagic spots, slightly raised. Extensive ecchymotic areas were found on the skin where the patient had applied mustard.<sup>13</sup> Jaundice marked; gums bleeding. Frequent emission of black vomit. The blood was transmitted to guinea pigs at 2.30 p.m., Oct. 4; condition becoming worse; anuria. Oct. 5, 3 p.m. Died.

*Autopsy.*—Performed 2 hours after death. Intense jaundice throughout the entire body. Skin over the chest showed numerous dark hemorrhagic spots. Lungs much congested, showing many hemorrhagic spots. Liver bright yellowish in color, friable, and fatty. Kidneys highly congested, with cloudy swelling; some small ecchymotic foci in the pelvis. Capsules not adherent. Stomach and intestines hyperemic with blackish bloody contents. Heart showed a few ecchymoses in the pericardium and endocardium; muscles degenerated. Bladder contracted with small amount of dark yellow urine. Spleen, pancreas, suprarenal glands, and testes apparently unchanged.

*Animal Inoculation.* *Guinea Pig 790 (Text-Fig. 2, d).*—Oct. 3, 1918, 2.32 p.m. Injected intraperitoneally with 3 cc. of blood from Patient Ch., on the 6th day of disease. Temperature rose to 40.2°C. on the 6th and 39.8° on the 7th day

<sup>13</sup> See Fig. 1, Paper I.



TEXT-FIG. 2, *a* to *d*. Direct transmissions from patient to guinea pig by means of injection of the blood of Patient Ch., Case 5. (*a*) Case 5. Age 30 years. (*b*) Guinea Pig 790 B. Strain, Case 5. (*c*) Guinea Pig 790 A. Strain, Case 5. (*d*) Guinea Pig 790. Strain, Case 5.

and came down to 37° on the 8th day, when the scleras and skin were intensely jaundiced. The animal was rapidly weakening and was killed for examination of the lesions and also for cultivation of material.

*Autopsy.*—Epistaxis; hemorrhagic lesions in the lungs and gastrointestinal tract. Liver pale yellow and fatty, and kidneys and suprarenal glands congested and swollen. Other organs apparently unchanged. In the blood and emulsions of the liver and kidneys the leptospiras were demonstrated. A pure culture was obtained from the blood of this animal.

*Guinea Pig 791.*—Duplicate of the foregoing experiment. The animal did not become infected.

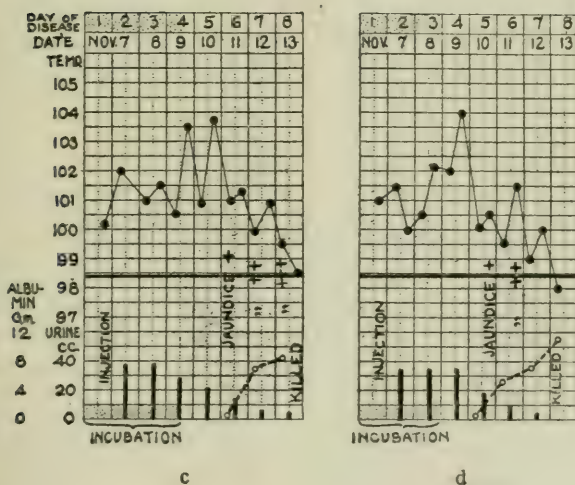
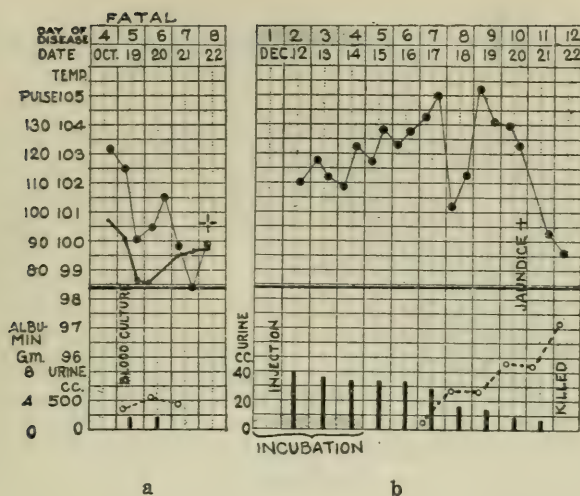
*Passage.*—Two guinea pigs were inoculated intraperitoneally on Oct. 11 with 1 cc. of blood from Guinea Pig 790, and both came down with typical symptoms and lesions within 8 days (Text-fig. 2, *b* and *c*). The leptospiras were demonstrated in the blood and emulsions of the liver and kidneys. Pure cultures were obtained from the blood of these guinea pigs.

The seventh positive transmission was obtained with a culture derived directly from the blood of Patient A. Ce.

*Case 6 (Text-Fig. 3, a).*—A. Ce., female, age 18 years; seamstress, from a coast town of Vines. Had been in Guayaquil 12 days.

Onset with chills and fever on Oct. 16, 1918, followed by headache, pains in the back, legs, and epigastric region. Vomited yellowish matter. Admitted on Oct. 18. Slight jaundice; marked albuminuria; intense pain throughout the body; nausea. Several petechial hemorrhages on the chest and arms. Oct. 19. Jaundice more distinct; tongue coated, with free edge and tip; bilious vomit; albuminuria; oliguria. Patient gravely ill. Blood culture made. Oct. 20. All symptoms becoming worse. Black vomit and profuse bleeding from the gums. Oct. 21. Comatose all day. Oct. 22, 8 a.m. Died. Autopsy not performed.

The culture tubes were examined on October 26, 1918, 7 days after they were set up, by the dark-field microscope. Only one of the six tubes showed the presence of active leptospiras in small numbers. From this twelve subcultures were made. The other tubes remained apparently without growth. Inoculations into guinea pigs were made at three different laboratories, first in the laboratory of the Yellow Fever Hospital in Guayaquil on October 27, then in the laboratory of the Colon Hospital, Panama Canal, on November 7, and finally in the laboratory of The Rockefeller Institute on December 11 and 19; that is, 8, 17, 44, and 52 days respectively after the time of making the cultures. Subsequent subcultures



TEXT-FIG. 3, *a* to *d*. Indirect transmission from patient to guinea pig by means of culture derived from the blood of Patient Ce., Case 6. (*a*) Case 6. Age 18 years. (*b*) Guinea pig. Strain, Case 6. Second passage from culture. (*c*) Guinea pig. Strain, Case 6. Culture 2. (*d*) Guinea pig. Strain, Case 6. Culture 3.



of this strain have since been obtained (Figs. 5 and 6). The protocols of some of these series of inoculations of guinea pigs with the cultures follow.

*Animal Inoculation (Second Series). Guinea Pig CeI<sub>2</sub> (Text-Fig. 3, c).—Nov. 7, 1918.* Inoculated intraperitoneally with 0.5 cc. of the culture (17 days old) of blood from Patient Ce. Temperature 39.8°C. on the 4th day, 40° on the 5th, 39° on the 6th, and 37.2° on the 7th. Suffusion of the conjunctivæ, ears, and soles. Slight jaundice appeared on the scleras, ears, and skin on the 7th day. The urine contained bile pigment and albumin. The animal was killed on Nov. 13 for examination and culture.

*Autopsy.*—Moderate general jaundice. Many spots of hemorrhage in lungs; liver congested and brownish dark red; kidneys swollen and cloudy; gastrointestinal mucosa hyperemic and somewhat hemorrhagic; spleen not altered.

*Guinea Pig CeI<sub>3</sub> (Text-Fig. 3, d).—The same as the foregoing experiment.* The animal showed symptoms and lesions almost identical with those just described.

*Animal Inoculation (Third Series). Guinea Pig 4, Second Passage from No. CeI<sub>2</sub> (Text-Fig. 3, b).—Dec. 11, 1918.* Inoculated intraperitoneally with 0.5 cc. of the liver emulsion of guinea pig infected with the first generation of culture of Ce. strain (35 days old) which had never been passed through any animal. Temperature 39.5°C. on the 4th, 39.8° on the 5th, 40.6° on the 6th, 38.8° on the 7th, 41° on the 8th, 40° on the 9th, 38° on the 10th, and 37.6° on the 11th day. The animal became intensely yellow throughout the entire body and was killed for culture and examination.

*Autopsy.*—All tissues were deeply jaundiced, and there were some minute ecchymoses in the subcutaneous and muscular tissues. The lungs showed large and small irregular hemorrhagic spots which on section were seen to extend into the parenchyma. The color of the liver was strikingly yellow. Kidneys swollen and on section cloudy; marking indistinct. Substance of kidney deeply jaundiced. Suprarenal glands congested. Mucosa of the gastrointestinal tract hyperemic. Some hemorrhages, staining the contents blackish red. Spleen not visibly affected. Only a few leptospiras found in the kidneys and none in the blood or liver.

In addition to the animals given above five more guinea pigs were inoculated in the same series. All showed symptoms and lesions similar to those just outlined. Fig. 4 shows the organism in a stained blood preparation from one of the infected guinea pigs.

The instance just described confirms the results obtained in the first positive transmission experiment (Case A.); namely, that the leptospira can be directly cultivated from the blood of yellow fever patients and then inoculated into guinea pigs with reproduction of

the symptoms and lesions which characterize the disease in man. As we shall note again elsewhere, the virulence of this organism was maintained in cultivation for more than 5 weeks.

To sum up the results recorded in the preceding series of experiments, it is shown that there exists in certain cases of yellow fever prevalent in Guayaquil a definite organism which is capable of being transmitted to the guinea pig and reproduces the symptom complex characteristic of yellow fever. As other experiments showed, the organism which has been observed and isolated in Guayaquil is a leptospira (Figs. 1 to 6) closely resembling the *Leptospira icterohæmorrhagiæ* of Inada and Ido,<sup>10</sup> discovered by them in the infectious jaundice of temperate climates. That the present organism is closely allied to but immunologically distinct from that species of the infectious jaundice has since been established and the experiments bearing on this point will be discussed in detail in subsequent papers. For the present I shall refer to the organism isolated from the yellow fever (typhus icteroides) cases as the yellow fever leptospira, or *Leptospira icteroides* (ἰκτερος, jaundice, + εἶδης, resembling, like), thus denoting the source from which it was obtained.

#### *Negative and Abortive Infections.*

In the twenty-one instances to be recorded there was either a temporary febrile reaction in the guinea pigs after inoculation of the blood, with or without any suspicion of jaundice, or almost no reaction at all. In other words, the results are classed as indefinite or negative. It is noteworthy, however, that a considerable proportion of the guinea pigs inoculated with the blood drawn during the first 5 days of the disease had a febrile reaction either on the 4th, 5th, 6th, or 7th day, and in some of these animals even a trace of jaundice was noted or suspected for a day or two soon after the fever. In none did the jaundice become definite, and the animals subsequently returned to normal. Undoubtedly, by transferring the virus to normal animals at this stage more strains might have been secured in passage, but much time was lost in watching further development of the symptoms, and opportunities to obtain the strains were lost. Some of these animals must have had a mild or abortive

TABLE I.

Case No.	Sex.	Age.	Course of disease.	Day of disease on which blood was taken.	No. of guinea pigs inoculated.	Outcome of inoculation.
		yrs.				
7	Male.	21	Severe; recovery.	2nd	2	Febrile reaction only.
8	"	21	" "	2nd	2	" " in one, fever and trace of icterus in other; recovery in both.
9	"	19	Moderate; "	2nd	2	No reaction in one, fever and trace of icterus in other; recovery in both.
10	Female.	26	Severe; died on 7th day.	2nd	2	No reaction.
11	"	10	Extremely mild.	2nd	2	" " in one, slight fever in other.
12	"	40	Moderate; recovery.	2nd	2	Febrile reaction in both.
13	Male.	35	Mild; "	3rd	2	" " " "
14	"	23	Severe; "	3rd	2	No reaction in one, fever in other.
15	"	25	Very mild; "	3rd	2	Fever in both.
16	"	25	Moderate; "	3rd	2	" " " and suspicion of icterus; both recovered.
17	"	19	Severe; died on 6th day.	3rd	1	Fever and suspected trace of icterus; recovered.
18	"	23	Severe; died on 7th day.	4th	1	Fever and suspicious yellow tint in scleras; recovered.
19	"	25	Severe; recovery.	4th	2	Febrile reaction in both and suspicion of icterus in one.
20	"	20	" "	4th	2	Fever in both and perhaps a trace of icterus.
21	"	28	Severe; died on 4th day.	4th	3	All had a febrile reaction but no icterus.
22	"	16	Severe; died on 10th day.	5th	2	Both had fever and suspiciously yellow scleras.
23	"	16	Moderate; recovery.	5th	1	Febrile reaction.
24	"	20	Severe; died in 6 (?) days.	5th(?)	2	Both had fever. Suspicion of icterus in one.
25	Female.	21	Mild; recovery.	6th	2	Fever in one, no reaction in other.
26	Male.	21	Severe; "	8th	2	No reaction in either.
27	"	32	" died on 16th day.	14th	2	" " " "
27	"	32	Severe; died on 16th day.	15th	2	" " " "

form of the infection, as they subsequently proved to be refractory to a virulent virus when tested after a period of about 25 days from the time of the inoculation of the yellow fever blood. For the sake of completeness the protocols of the negative transmission experiments are recorded in Table I.

#### SUMMARY.

By injecting into guinea pigs the blood of yellow fever cases occurring in Guayaquil a group of symptoms and lesions closely resembling those observed in human yellow fever were induced in a limited number of instances. Of 74 guinea pigs inoculated with specimens of blood from 27 cases of yellow fever, 8, representing 6 cases, came down with the symptoms; namely, a marked rise of temperature after a period of incubation averaging 3 to 6 days, with simultaneous suffusion of the capillaries, particularly of the conjunctivæ and soles, then preliminary hyperleucocytosis followed by progressive leucopenia, the early appearance of albumin and casts in the urine, which gradually diminishes in volume as the disease progresses. The fever lasts only a few days, rapidly dropping first to the normal and then usually to subnormal. At this period jaundice manifests itself in varying degrees of intensity, first in the scleras, then in the skin and the urine. Hemorrhages from the nasal or gingival mucosa or anus have been observed to occur during this period. Autopsies reveal deep jaundice throughout the entire tissue. The liver is fatty and yellow, the kidney hyperemic, and often swollen and hemorrhagic. Hemorrhagic spots were almost always found in the lungs and gastrointestinal mucosa. Guinea pigs are usually rather sensitive to the infection, though many appeared to be somewhat resistant and some even refractory.

The injection of the yellow fever blood into ringtail monkeys, rabbits, cats, guatusas, weasels, and sloths among the mammals, and pigeons, ground-doves, bluebirds, mantas, blackbirds, parakeets, reedbirds, blancos, and toucans among the birds, gave negative results.

In the blood, liver, and kidneys of the guinea pigs experimentally infected with the blood of yellow fever patients a minute organism



was demonstrated which closely resembles in morphology the causative agent of infectious jaundice (*Leptospira icterohæmorrhagiæ*).

The leptospira transmitted from yellow fever cases to guinea pigs was found to induce similar symptoms and lesions upon further passage into normal guinea pigs.

The leptospira obtained from cases of yellow fever has been given the provisional name of *Leptospira icteroides*.

I wish to express my thanks to Dr. León Becerra, General Director of the Department of Health of Ecuador, and his staff for their support and cooperation in the execution of this work; and likewise to Dr. Pareja of the Guayaquil Yellow Fever Hospital and to his laboratory staff (Dr. Larrea), clinical staff (Dr. Davila and Dr. Martinez), and nursing staff for their invaluable assistance.

To Dr. Herman B. Parker I am indebted for his courtesy in lending me a dark-field arc lamp during the period before my own arrived, when the dark-field work would otherwise have been impossible, and also in furnishing me on various occasions with clinical material.

I am indebted to Colonel McCormack, Chief Health Officer of the Canal Zone, to Major Teague and Captain McFarland of the Ancon Hospital, and to Captain Bowen and Lieutenant Levy of the Colon Hospital, for the facilities afforded me in making renewal of cultures and transfers of strains to new animals.

#### EXPLANATION OF PLATE 35.

FIG. 1. *Leptospira icteroides* in the blood of a guinea pig experimentally inoculated with culture of strain from Patient A. A., Case 1. Fixed in methyl alcohol and stained with Wright's stain. The film was made on the 6th day of illness.  $\times 1,000$ .

FIG. 2. The same; strain from Patient M. G., Case 3.  $\times 1,000$ .

FIG. 3. The same; strain from Patient Co., Case 4.  $\times 1,000$ .

FIG. 4. The same; strain from Patient A. Ce., Case 6.  $\times 1,000$ .

FIG. 5. Dark-field view of a culture 16 days old of *Leptospira icteroides*. Strain from Patient A. Ce., Case 6.  $\times 1,000$ .

FIG. 6. The same.  $\times 1,000$ .

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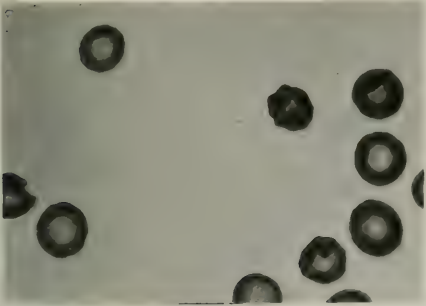


FIG. 1.

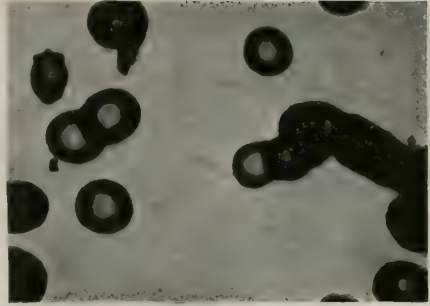


FIG. 2.

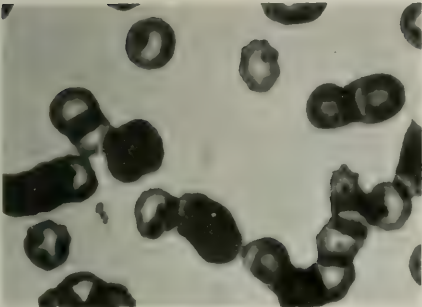


FIG. 3.

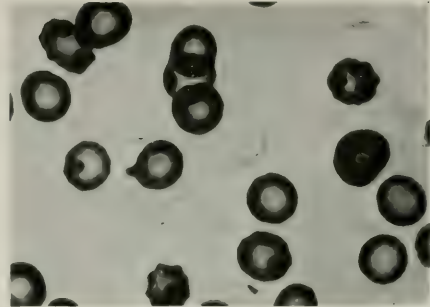


FIG. 4.

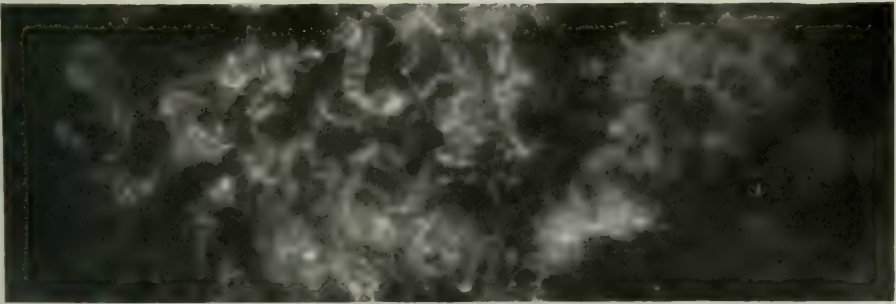


FIG. 5.

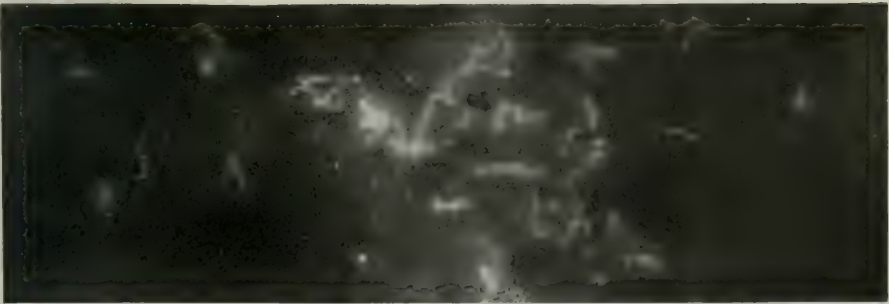


FIG. 6.



## ETIOLOGY OF YELLOW FEVER.

### III. SYMPTOMATOLOGY AND PATHOLOGICAL FINDINGS IN ANIMALS EXPERIMENTALLY INFECTED.

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PLATES 36 TO 38.

(Received for publication, March 26, 1919.)

Mention has already been made<sup>1</sup> of successfully inducing in guinea pigs symptoms and pathological conditions simulating those observed in yellow fever patients in Guayaquil by inoculating these animals with the blood or organ emulsions from yellow fever patients. It has also been stated<sup>1</sup> that in the blood and organ emulsions of the infected guinea pigs an organism belonging to the genus *Leptospira* has been demonstrated and that the organism, after having been obtained in culture, is capable of inducing the same symptoms and pathological changes in these animals as does the original blood of yellow fever patients. In this paper the mode and course of this infection as observed in guinea pigs, dogs, and monkeys will be described.

#### *Mode of Experimental Infection.*

Infection with this organism may be induced either by injection into the peritoneal cavity, the blood circulation, or the subcutaneous tissues, or by application to the scarified, depilated surface of the skin or to the mucous membranes, or by feeding the animal with infected tissue or culture.

#### *Experimental Infection in Guinea Pigs (Text-Fig. 1, a to f).*

*Incubation Period.*—The incubation period varies according to the mode of infection and the quantity of virus introduced. When a large

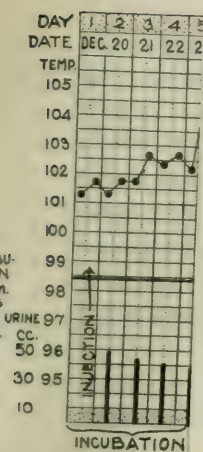
<sup>1</sup> Noguchi, H., Etiology of yellow fever. II. Transmission experiments on yellow fever, *J. Exp. Med.*, 1919, xxix, 565.



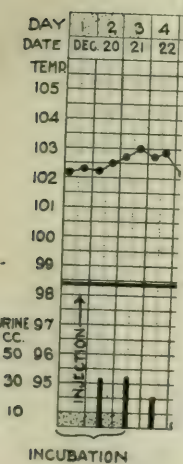
amount is inoculated intraperitoneally or into the circulation the first symptoms—inactivity, anorexia, hyperleucocytosis—make their appearance after 48 hours, followed by a rise in temperature and slight albuminuria within the next 24 hours. With subcutaneous inoculation the symptoms do not appear until the 4th (72 hours) or 5th (96 hours) day, and with a very small amount of the virus a few days later. The percutaneous and *per os* modes of infection require a period of about 6 and sometimes as many as 12 days of incubation.

*Onset and Course.*—The onset is indicated by loss of appetite, inactivity, injection of the bulbar conjunctivæ, and a sudden rise in temperature ranging from 39.8–40.5°C. and rarely 41°C. The animal offers little resistance to handling, plaintive cries indicating intense muscular pains. The urine diminishes in quantity from an average daily output of about 25 cc. to almost half that, and its color changes from a pale straw tinge to dark brownish yellow. It now contains a moderate amount of albumin, with some epithelial cells and granular casts. At this stage no bile pigment can be demonstrated in the blood serum and no jaundice in the scleras or other parts of the body. The leucocytes are increased during the 1st day, but leucopenia follows within a few days, the differential count showing a marked increase of polymorphonuclear leucocytes.<sup>2</sup> Within the next 24 hours the temperature shows a slight drop, only to return to 39.8–40°C. during the following day. The fever then subsides gradually until the temperature drops below the normal in 3 or 4 days more. In fatal cases it sometimes drops to 34°C. just before death. Although the temperature begins to fall on the 2nd day all the other symptoms are aggravated. The urine is reduced still further in quantity and contains an enormous amount of albumin, renal epithelia, granular and hyaline casts, and the bile pigment first appears. At the same time the scleras become deeper yellow and are intensely suffused. At this stage there is a trace of bile pigment in the serum. On the 3rd day icterus becomes quite distinct

<sup>2</sup> I am greatly indebted to Dr. Jorge Larrea, Director of the Laboratories of the Guayaquil Yellow Fever Hospital, for total leucocyte counts on some of the experimental animals. My thanks are also due to Dr. F. Rojas of the General Hospital in Guayaquil.



a



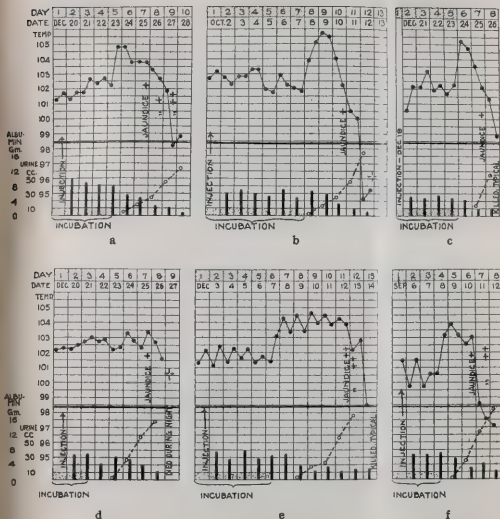
b

TEXT-FIG. 1, a to f, showing the course of the disease in strains of *Leptospira*. (a) Case 1. (b) Case 2. (c) Guinea pig, Case 3. (d) Case 4. (e) Case 5. (f) Case 6. (f) Case 6.

ecchymoses in the feet but is and the serum Erythrocytes, indant in the e total anuria. l, and epistaxis Death occurs disease and is l asphyxiation, w that about flows from the across.

yellow fever in ion of the ani- w a temporary idice is slightly t in color) and ever and other away within a revious health. is of the same cannot be rein- to kill control

Furthermore, elated with the n among others ently when the rain attenuated man, therefore, ere apparently ng to the indi- ces have been s purchased in mmunity to the



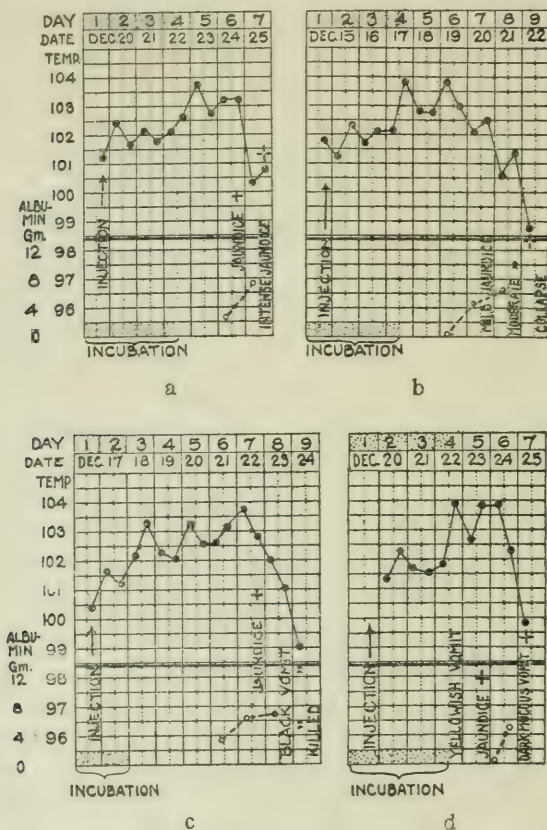
TEXT-FIG. 1, a to f. Observations on guinea pigs inoculated with the cultures of the different strains of *Leptospira icteroides*. (a) Guinea pig. Strain, Case 5. (b) Guinea pig. Strain, Case 5. (c) Guinea pig. Strain, Case 6. (d) Guinea pig. Strain, Case 5. (e) Guinea pig. Strain, Case 6. (f) Guinea pig. Strain, Case 1.

in the ears, scleras, palms, and soles. There may be ecchymoses in the scleras. Epistaxis and hematuria are frequent symptoms. The urine is now a deep grayish brown hue, and only a few cubic centimeters may be excreted. The animal still keeps on its feet but is quiet. On the 4th day the jaundice becomes general, and the serum taken is deep brownish yellow, as is also the urine. Erythrocytes, renal cells, albumin, and bile pigment are very abundant in the scanty urine which may still be passed, or there may be total anuria. Melena or bleeding from the rectum has been observed, and epistaxis is a frequent phenomenon in a dying animal (Fig. 13). Death occurs between the 5th and 7th days after onset of the disease and is accompanied either by clonic convulsions or by gradual asphyxiation, with air-hunger. The blood pressure becomes so low that about 2 days before death very little or almost no blood flows from the largest ear veins, even when they are completely cut across.

*Non-Fatal and Abortive Infections.*—Experimental yellow fever in the guinea pig is not always fatal. A certain proportion of the animals inoculated with a virulent strain of the virus show a temporary febrile reaction and albuminuria with few casts. Jaundice is slightly noticeable in the ears (if the animal is white or light in color) and scleras, but it is sometimes apparently absent. The fever and other symptoms set in as in fatal cases but completely pass away within a few days. In a week the animal has regained its previous health. That these reactions are mild or atypical symptoms of the same infection is evident from the fact that such animals cannot be reinfected, even with a quantity of the virus sufficient to kill control guinea pigs with the typical symptoms and lesions. Furthermore, these mild infections occur among guinea pigs inoculated with the same quantity of virus which produces a fatal infection among others in the same group, and especially do they occur frequently when the amount of the virus injected is rather small or the strain attenuated in virulence for the guinea pig. As in yellow fever in man, therefore, so in the experimental condition in guinea pigs, there apparently exist varying grades of severity of infection according to the individual resistance to the same virus. Many instances have been encountered, among many hundreds of guinea pigs purchased in Guayaquil, in which the animals showed a complete immunity to the



inoculation of the virus. It is possible that as some of them had been kept in houses or markets for varying periods of time before purchase they were rendered immune through a previous mild infection.



TEXT-FIG. 2, a to d. Observations on dogs inoculated with the cultures of the different strains of *Leptospira icteroides*. (a) Dog (pup). Strain, Case 6. (b) Dog (pup). Strain, Case 1. (c) Dog (pup). Strain, Case 5. (d) Dog (pup). Strain, Case 3.

*Experimental Infection in Dogs (Text-Fig. 2, a to d).*

*Incubation Period.*—This is practically the same as in guinea pigs.

*Onset and Course.*—The onset and course are also similar to those observed in guinea pigs, some dogs succumbing to the infection and

others recovering from a mild attack. In a fatal infection the temperature may reach 40°C., but usually it is not over 39.5°C., and lasts for 3 or 4 days, when it gradually drops by lysis. The skin then becomes yellowish and the conjunctivæ injected, and the animal refuses to eat. It often vomits bilious and sometimes blackish, mucous, frothy contents from the stomach. The amount of urine diminishes, with increasing albumin, casts, and bile pigments. Death occurs in coma or in clonic convulsions, the temperature falling below normal.

*Experimental Infection in Monkeys (Text-Fig. 3, a to e).*

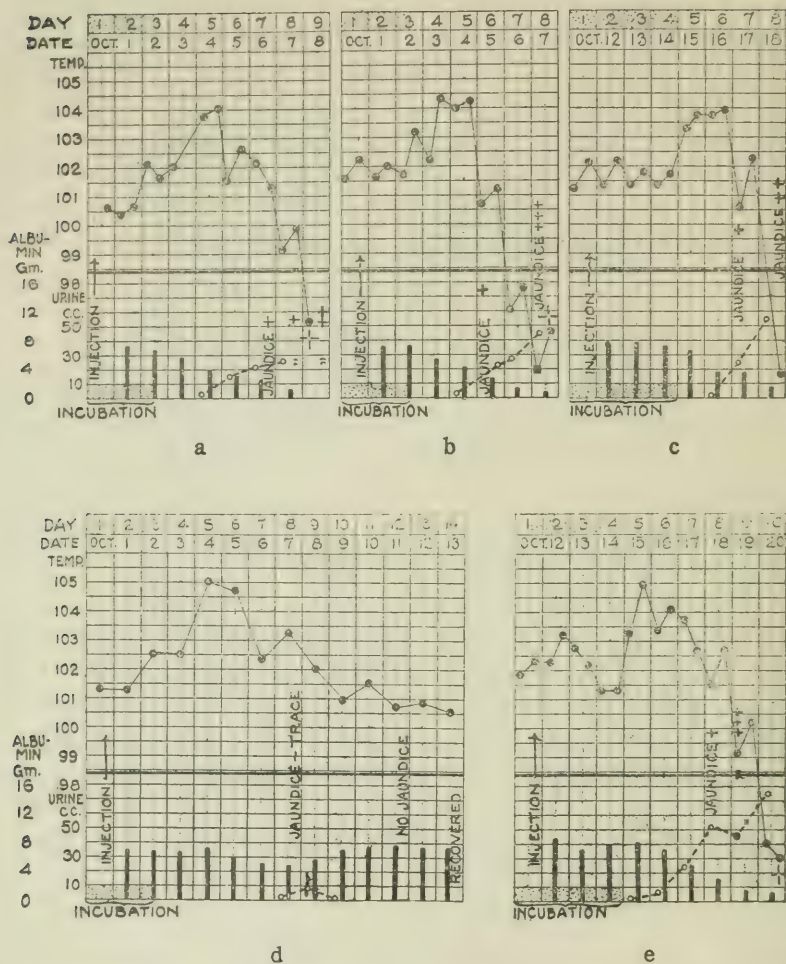
As stated elsewhere, the South American ringtail monkeys were found refractory to infection with the blood of yellow fever patients or the culture of the yellow fever leptospira.<sup>1</sup> With both a temporary febrile reaction was observed, but the animal invariably recovered. Five marmosets,<sup>3</sup> however, proved to be susceptible to the organism, four being fatally and one mildly infected.

The incubation period varied from 3 to 4 days, during which time the animals appeared well. The disease became noticeable by their inactivity, loss of appetite, non-resistance to handling, and slight greenish pigmentation of the urine. Albumin, casts, and bile pigments began to appear in the urine when the fever reached its height. Mild jaundice in the scleras, abdominal skin, and oral mucous membrane became noticeable a day or two after the fever began to subside. On the day of death the animals lay in a helpless position, offering only feeble resistance to handling. Death occurred during a condition of general weakness, coma, and sometimes convulsions. The temperature was usually subnormal, and the body of the animal more distinctly and generally yellowish.

*Autopsy Findings in the Experimental Infection (Fig. 14).*

The guinea pigs, dogs, and marmosets dying of the experimental infection invariably show a pronounced jaundice ranging from a light yellow to a deep bright yellow. Blood clots or stains are often

<sup>3</sup> Species *Midas adipus* and *Midas geoffroyi*.



TEXT-FIG. 3, *a* to *e*. Observations on monkeys inoculated with the different strains of *Leptospira icteroides*. (*a*) Monkey 1. Strain, Case 1. Culture of second generation, 2 cc. intraperitoneally. (*b*) Monkey 2. Strain, Case 3. Culture of first generation, 4 cc. intraperitoneally. (*c*) Monkey 5. Strain, Case 6. Guinea pig kidney emulsion, 6 cc. intraperitoneally. (*d*) Monkey 3. Strain, Case 1. Culture of second generation, 4 cc. intraperitoneally. (*e*) Monkey 4. Strain, Case 5. Guinea pig liver emulsion, 6 cc. intraperitoneally.

present in the nostrils, mouth, anus, or vagina. Subcutaneous ecchymoses are frequently present in the guinea pig but seldom in the dog or marmoset. Postmortem rigor and lividity are marked. In guinea pigs the subcutaneous tissues are intensely jaundiced, and ecchymoses are seen in the axillary and inguinal regions. The abdominal muscles are often spotted with minute ecchymoses but sometimes these are absent. In the dog and marmoset the muscles show very slight or no hemorrhage.

The lungs of the guinea pig almost always show ecchymoses varying in extent from few and minute to numerous and large ones, roughly round, oval, or irregularly oblong or square, and with or without a sharp outline, vivid red, dark, or bluish red in color, sometimes light red with a darker center. Undoubtedly the color of the spots becomes darker and more bluish as they become older, since the longer the animal lives the darker are these ecchymoses. Postmortem hypostasis is marked. There may be some ecchymoses in the pleuræ. In the dog and marmoset the lungs are much less affected, only a few spots of hemorrhage being found, and the pleuræ are usually free.

There may be some dilation of the right heart. The pericardium is often studded with minute hemorrhagic spots. The fluid is clear and icteric. The muscle is friable and brownish yellow, and there are few or sometimes many ecchymoses on the surface. The endocardium and papillary muscles seem normal except for occasional punctiform ecchymoses. The valves are not altered but are yellowish in color, the aorta usually being deeply jaundiced. These changes apply equally to the guinea pig, dog, and marmoset.

The liver is usually slightly enlarged and varies in color from a yellowish brown-red to a bright yellowish brown. In instances in which death occurred within the first 3 days the degeneration was less advanced and the yellowish color not so pronounced. The surface is often mottled or striped with yellowish brown and brownish red, and the markings are very distinct.

The gall bladder is usually filled with a dark green or greenish yellow bile, and the wall is often spotted with minute ecchymoses.

The stomach usually contains some undigested food which is mixed with blood from the adjacent ecchymotic area of the mucosa. The contents are sometimes semifluid containing blackish blood re-



sembling coffee-grounds in appearance. In dogs and marmosets the greater quantity of mucus renders the appearance of the stomach contents indistinguishable from those found in human autopsies. The mucosa is somewhat hyperemic, and numerous ecchymoses are found, especially near the cardia. The serosa of dogs and marmosets is free of hemorrhages.

The small intestine and colon, including the rectum, are intensely injected, and numerous hemorrhages are found in the mucosa. The serosa is sometimes affected. The contents are blackish in color and may contain freshly escaped blood (melena). In the dog the character of the intestinal contents is a closer reproduction of what is observed in man, the serosa in this animal not showing the ecchymoses which are present in the guinea pig or any appreciable fluid in the peritoneal cavity. The nature of the findings in marmosets is about midway between that of guinea pigs and that of dogs.

In guinea pigs the kidneys are extensively involved. Hyperemia and punctiform hemorrhages in the parenchyma are almost constant. The ecchymoses, however, vary from a few to almost countless numbers in extreme instances. In dogs and marmosets there may be only a few minute spots. On section the cortex is broader than normally and highly hyperemic, with cloudy swelling. The medullary portion is succulent and hyperemic near the border. Bloody fluid or clot may be found in the pelvis, and sometimes numerous punctiform ecchymoses.

In the guinea pig hyperemia and hemorrhages were found in the suprarenal glands, but in dogs and marmosets only a comparatively slight degree of hyperemia. There were no changes in the pancreas or spleen, except a slight enlargement of the latter in rare instances.

The lymphatic glands show, in guinea pigs, general adenopathy, with occasional hemorrhages. In dogs and marmosets some glands only are enlarged and congested.

The bladder frequently contains bile-stained urine full of albumin, casts, and cells.

The testicles are apparently not affected, although in the guinea pig hemorrhages are frequent in the adipose tissue around them.

The ovaries are usually congested, and the uterus very much so, and in the pregnant state there were hemorrhages into the amnionic

fluid. The endometrium is congested, sometimes with a clot in the cavity.

No gross changes are observed in the nervous system. The membranes are hyperemic and the fluid is icteric.

*Histological Examination (Figs. 1 to 12).*

*Lungs (Figs. 3 and 6).*—The areas of hemorrhage are most abundant and extensive in the guinea pig. The alveoli in the hemorrhagic areas are completely filled with blood corpuscles, and in the adjacent zone a marked degree of edema is evident. There are also small accumulations of polymorphonuclear leucocytes and endothelial cells. In marmosets and dogs these changes are far less extensive. The leptospiras were demonstrated in the tissues by the Levaditi method.

*Liver (Figs. 1 and 4).*—The degree of degeneration of the liver cells is variable in different animals. In well marked instances the majority of the cells are swollen and necrotic. Vacuoles are found in some cells. The nuclei are swollen and degenerated. In some areas the liver cells are dissociated, swollen, and appear to have lost their sharp hexagonal outlines. The liver cells near the blood vessels seem to be less affected than those around the portal canal. Hemorrhagic foci of varying dimension are distributed irregularly. The endothelial cells of the bile ducts are increased in size, and there are some lymphoid cells around the portal canal zone. Mitotic figures are found. The organisms were found in the tissues stained by Noguchi's method (Figs. 7 and 10).

*Kidneys (Figs. 2 and 5).*—The epithelium of the convoluted tubules shows granular, swollen, and sometimes vacuolated cytoplasm. The cells may be detached from the membrane and fill up the lumen, which is distended with granular and hyaline casts. The glomeruli are considerably injected, and numerous hemorrhages are found throughout the cortex and medulla (Figs. 8, 9, 11 and 12).

*Stomach.*—There are superficial congestion of the mucosa and some hemorrhagic foci. In certain areas there is an accumulation of lymphoid and plasma cells.

*Large and Small Intestines.*—The intestines show injection and occasional hemorrhages.

*Heart*.—Certain fibers are swollen and contain vesicular nuclei.

*Spleen*.—There are hemorrhages, and the pulp is rich in blood.

*Lymph Nodes*.—These show phagocytosis and central degeneration of the follicles.

*Adrenal*.—In guinea pigs there are parenchymatous degeneration, congestion, and hemorrhages in some instances. These changes are less frequent in dogs and marmosets.

*Pancreas*.—Little change.

*Nervous System*.—Little change.

#### SUMMARY.

Studies are reported on the type of disease induced in guinea pigs, dogs, and monkeys by inoculating them (1) with the blood or organ emulsions of guinea pigs or other susceptible animals experimentally infected with *Leptospira icteroides*, and (2) with a pure culture of the organism. Particular attention has been given in these experiments to the clinical features of the experimental infection in the various animals and to the pathological changes resulting from the infection.

The symptoms and pathological lesions induced in guinea pigs are much more pronounced than those observed in dogs or marmosets. The period of incubation is nearly the same in all three species, 72 to 96 hours with intraperitoneal or subcutaneous inoculation, and a day or more longer when the infection is induced percutaneously or *per os*. The febrile reaction in the guinea pig and marmoset is about the same; in the dog there is less fever. The amount of albumin, casts, and bile pigments in the urine is more abundant in the guinea pig and marmoset than in the dog, and these animals also appear on the whole to become more intensely icteric. The black or bilious vomit, however, though occurring frequently in dogs during life, is observed in the guinea pig and marmoset at autopsy. The hemorrhagic diathesis is most pronounced in guinea pigs, less so in marmosets, and least in dogs. In dogs, for example, subcutaneous hemorrhages almost never occur, and the lungs usually show only a few minute ecchymoses. The pleuræ, pericardium, and other serous surfaces of the thorax and abdomen remain free from ecchymoses,

which, however, with hyperemia, are very marked along the gastrointestinal tract.

The symptoms and lesions observed in animals experimentally infected with *Leptospira icteroides* closely parallel those of human yellow fever.

The pathological changes occurring in human cases of yellow fever are similar to those induced by inoculation in guinea pigs and marmosets and in respect to their intensity stand intermediate between those arising in the two animals mentioned.

#### EXPLANATION OF PLATES

##### PLATE 36.

Sections of tissues from yellow fever cases, fixed in Zenker's fluid and stained with eosin and methylene blue.  $\times 150$ .

FIG. 1. Section of the liver of a marmoset inoculated with the organism isolated from Patient E. Ch., Case 5, showing hemorrhage and necrosis, vacuolization, and dissociation of the liver cells.

FIG. 2. Section of the kidney from the same marmoset, showing necrosis, detachment, and some vacuolization of the renal epithelia. Some of the lumina are seen to contain granular casts. The glomeruli seem to be highly congested; hemorrhage in some portions of the tissue.

FIG. 3. Section of the lung from the same marmoset, showing hemorrhage in the tissue.

FIG. 4. Section of the liver from a guinea pig inoculated with the organism isolated from Patient M. G., Case 3. The liver cells are seen to have been largely disintegrated and replaced by red corpuscles and debris.

FIG. 5. Section from the kidney of same guinea pig, showing advanced degeneration of renal epithelia, with granular and hyaline casts in the lumen.

FIG. 6. Section of the lung from a guinea pig inoculated with the strain isolated from Patient A. Ce., Case 7. There is a considerable degree of hemorrhage and edema. Some leucocytic infiltration seems to be evident.

##### PLATE 37.

Sections from the liver and kidney of marmosets and guinea pigs inoculated with the strains of *Leptospira icteroides* derived from Cases 1, 3, and 5. Figs. 10 to 12 are stained by the method of Levaditi, Figs. 7 to 9 by the writer's modification of that method.  $\times 1,000$ .

FIG. 7. Liver of marmoset, showing the organism between the hepatic cells; strain from Case 5.



FIG. 8. The same strain. There are two minute, delicate organisms in the lumen of a kidney tubule.

FIG. 9. The same.

FIG. 10. The organism in the guinea pig liver, in which it appears somewhat coarse.

FIGS. 11 and 12. The organism in the kidney; coarse and quite numerous.

#### PLATE 38.

FIG. 13. Epistaxis in a guinea pig inoculated with a culture of *Leptospira icteroides* isolated from Patient A. Ce., Case 6. The picture was taken at the moment of death.

FIG. 14. Autopsy of a guinea pig killed on the 6th day after inoculation with a culture of *Leptospira icteroides* isolated from Patient A. Ce., Case 6. The animal showed typical symptoms and was killed at the time of collapse. The picture shows the general jaundice of the skin and subcutaneous tissues and the yellowish liver. Hemorrhages are evident in the lungs, and can be seen to have taken place in the stomach, as shown by its dark color. The kidney is congested, and sometimes a few ecchymotic spots can be seen. The spleen appears to be normal. The intestines and also the muscles along the thorax, abdomen, and postperitoneal region are comparatively free from hemorrhage, presenting a striking contrast to the highly congested condition of these muscles in guinea pigs inoculated with the organism of infectious jaundice. The strikingly yellow color of the liver is far more intense than is usually observed in experimental infectious jaundice in the guinea pig.

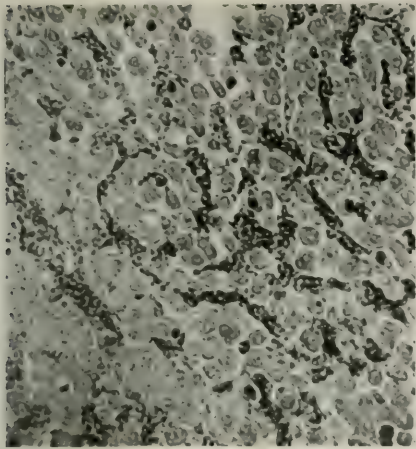


FIG. 1.

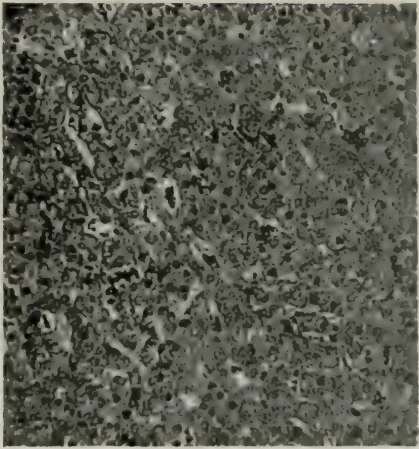


FIG. 4.

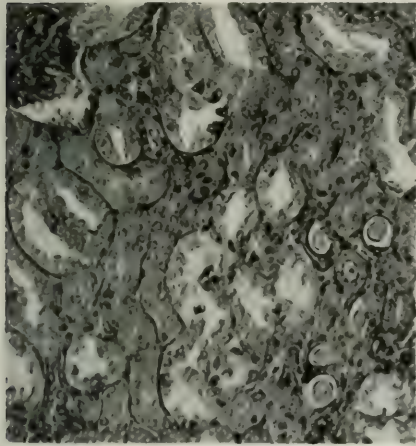


FIG. 2.

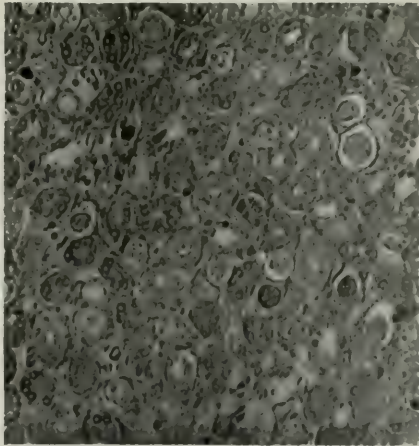


FIG. 5.

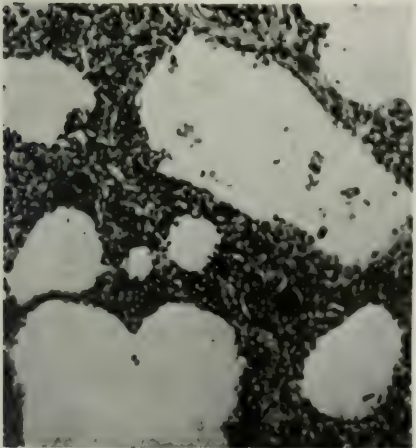


FIG. 3.

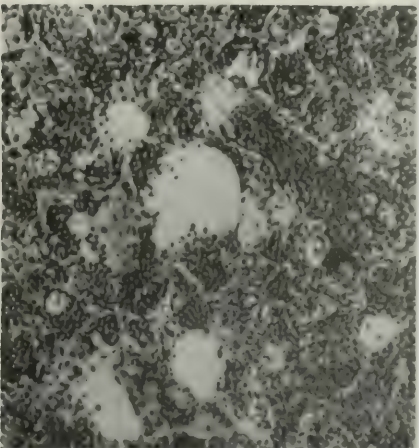


FIG. 6.



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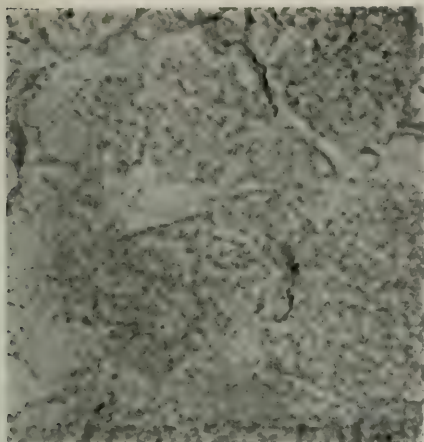


FIG. 7.

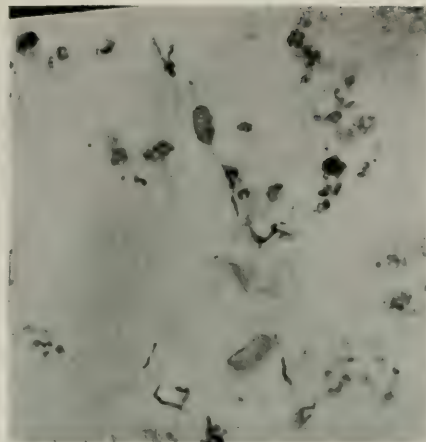


FIG. 10.

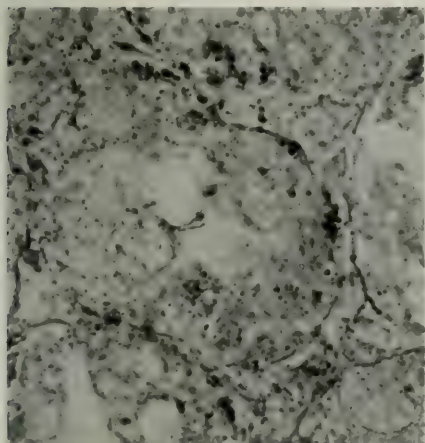


FIG. 8.

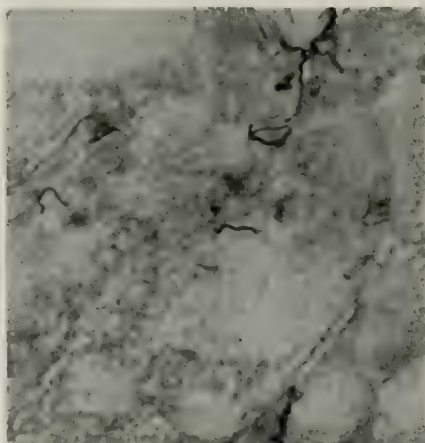


FIG. 11.

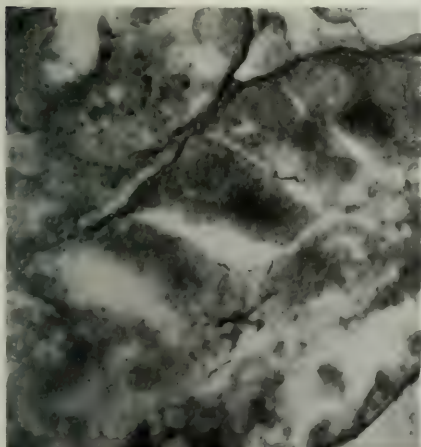


FIG. 9.

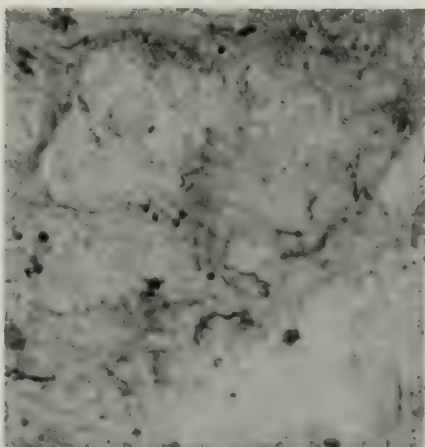


FIG. 12.







FIG. 13.



FIG. 14.



## ETIOLOGY OF YELLOW FEVER.

### IV. THE ACQUIRED IMMUNITY OF GUINEA PIGS AGAINST LEPTOSPIRA ICTEROIDES AFTER THE INOCULATION OF BLOOD OF YELLOW FEVER PATIENTS.

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(Received for publication, May 2, 1919.)

Most of the attempts to transmit yellow fever by inoculating the blood of patients into various animals have ended in failure. As stated elsewhere<sup>1</sup> out of 74 guinea pigs, in only eight were the results positive, and in other species of animals they were all negative. It should be emphasized that for the purpose of transmission cases were selected which were still in the early stage of the disease, previous investigators having pointed out the fact that the virus may no longer exist in the peripheral blood after the 3rd day of the illness.

In the present study the guinea pigs which failed to manifest the typical symptoms, or any symptoms at all, were kept under daily observation for many weeks. On a later date, through temporary shortage of normal guinea pigs, they were inoculated, together with several normal guinea pigs, with a virulent organ emulsion, rich in the leptospira, from a guinea pig experimentally infected with the organism, in order to prepare a large amount of organ emulsion for the purpose of immunizing two donkeys against the virus. A great many of the guinea pigs remained perfectly well, while most of the normal animals, as well as those which had been previously inoculated with the blood of malarial patients,<sup>2</sup> came down with an experimental infection.

<sup>1</sup> Noguchi, H., *J. Exp. Med.*, 1919, **xxix**, 565.

<sup>2</sup> In a country where æstivo-autumnal parasites infest the population many times a malarial patient is brought to the Yellow Fever Hospital on the chance that the case is one of early yellow fever. All these cases were used for the experiments on the transmission of yellow fever without the loss of time which would have been caused by waiting for a diagnosis, for which a day or two might be required.



TABLE I.  
*Susceptibility of Guinea Pigs to the Inoculation of Leptospira icteroides 25 Days after They Had Been Injected with the Blood of Yellow Fever Patients.*

Patient.	Sex.	No. of guinea pig.	Day of disease when bled.	Amount of blood injected.	Reaction.	Subsequent test for susceptibility.	Reaction.	Result.
Case 27. Yellow fever. Died in 16 days.	M.	16	14th	cc. 2	None.	Virulent Arias strain after 25 days.	40.3 in 5 days.	Killed in 8 days. Typical.
		17	14th	3	"	"	39.8 " 6 "	Died in 8 days. Typical.
Case 25. Yellow fever. Mild. Recovery. Also malaria.	F.	21	6th	3	"	"	39.9 " 6 "	Died in 8 days. Typical.
		22	6th	3	39.7 in 4 days.	"	None.	Remained well.
Case 1. Malaria (Guayaquilian).	"	23		3	None.	"	40.5 in 8 days.	Killed in 11 days. Mild but typical.
		24		4	"	"	40.0 " 7 "	Killed in 9 days. Typical.
Case 27. Yellow fever. Second specimen of blood.	M.	32	15th	5	"	"	39.9 " 5 " Icterus.	Killed in 7 days. Typical.
		33	15th	5	"	"	40.0 in 6 days. Icterus.	Died in 8 days. Typical.
Case 13. Dr. Valenzuela's patient. Yellow fever. Mild. Recovery.	"	35	3rd	3	39.8 in 8 days.	"	40.2 in 4 days.	Recovered.
		36	3rd	5	39.2 " 12 "	"	39.6 " 5 "	Remained well.



TABLE I—*Concluded.*

Patient.	Sex.	No. of guinea pig.	Day of disease when bled.	Amount of blood injected.	Reaction.	Subsequent test for susceptibility.	Reaction.	Result.
Case 22. Yellow fever Died in 10 days.	M.	198	5th(?)	cc. 3	°C. 40.0 in 4 days. Trace of icterus (?).	Virulent Arias strain after 25 days.	None.	Remained well.
		199	5th	3	40.0 in 7 days. Trace of icterus (?).	" "	"	"
Case 19. Yellow fever. Severe. Recovery.	"	200	4th(?)	3	39.6 in 4 days.	" "	"	"
		201	4th	3	40.0 " 5 Trace of icterus.	" "	"	"
Case 20. Yellow fever. Severe. Recovery.	"	220	4th(?)	3	40.3 in 7 days. Trace of icterus (?).	" "	"	"
		221	4th	3	40.2 in 5 days. Trace of icterus (?).	" "	"	"
Case 16. Yellow fever. Severe. Recovery.	"	222	3rd(?)	3	40.1 in 7 days. Trace of icterus (?).	" "	"	"
		223	3rd	3	40.3 in 6 days. Trace of icterus (?).	" "	"	"

Case 28. Yellow fever. Died.	M.	329	(?) Brought in unconscious. Died same day.	5	None.	Virulent Arias strain after 25 days.	40.0 in 5 days Icterus.	Died in 7 days Typical.
Case 10. Yellow fever. Died in 7 days.	F.	338	2nd	4	"	"	40.1 in 6 days Icterus.	Killed in 8 days. Typical.
		339	2nd	4	"	"	39.5 in 7 days Icterus.	Killed in 10 days. Typical.
Case 24. Yellow fever. Died.	M.	340	(?) Died next day.	4	39.2 in 5 days. No icterus. Died in 8 days; hemorrhages.			
		341	" "	4	40.0 in 13 days. No icterus.	"	40.0 in 6 days Icterus.	Died in 9 days. Typical.
Case 8. Yellow fever. Severe. Recovery.	"	342	2nd	4	40.0 in 12 days. No icterus.	"	40.2 in 7 days Icterus.	Died in 10 days. Typical.
		343	2nd	4	39.6 in 6 days. Trace of icterus.	"	None.	Remained well.
Case 18. Yellow fever. Died in 7 days.	"	407	4th	3	39.8 in 5 days. Trace of icterus.	"	"	"



Apparently some of the guinea pigs previously inoculated with the blood from yellow fever cases were refractory to the subsequent inoculation with a known virulent organ emulsion. As shown in Table I, those which resisted the infection were found to have had a febrile reaction of varying degree and duration on about the 5th day after the injection of the blood. This seemingly unimportant fever reaction must have been the result of a very mild abortive infection totally unsuspected on account of the absence of the main symptom, the jaundice. This fact is proved, in my opinion, by the acquired resistance of an animal which, when a sufficient quantity of a passage strain of *Leptospira icteroides* is introduced, is so susceptible. There is reason to believe that the strains of *Leptospira icteroides* as they occur in man are on the whole less virulent to the guinea pig and are incapable of producing a fatal infection from the beginning except in rare instances. It may be recalled here that with one strain (Case 2) the organism did not become thoroughly adapted to the guinea pig until it had been passed through three generations in this animal.<sup>1</sup>

A complete, or nearly complete refractoriness or resistance to *Leptospira icteroides* was demonstrated in sixteen guinea pigs previously inoculated with the blood of yellow fever patients, representing nine out of fourteen cases of yellow fever. Four out of six guinea pigs injected with the blood of two other yellow fever patients died, and two survived. In this group the protection was present in some of the guinea pigs only. In a doubtful case one of the two guinea pigs resisted a subsequent infection. On the other hand, of six guinea pigs, which received the blood of three patients, all died with the typical symptoms and lesions when tested later with a virulent passage strain of *Leptospira icteroides*. Of ten, which received injections of blood from four malarial patients, all proved to be susceptible to a later inoculation with the same strain of *Leptospira icteroides* that was used in the foregoing experiments.

Of the guinea pigs representing the yellow fever group, those which had had a febrile reaction several days after the injection of the blood are the ones which acquired the immunity against the inoculation of *Leptospira icteroides*. There seems to exist some relation between the febrile reaction and the acquired immunity. In several instances, moreover, these guinea pigs showed a suspicious trace of

jaundice in the scleras some time after the height of fever had passed, but the disease had failed to advance further. If the animals had been killed at the proper time and transfers made to normal guinea pigs the virulence of the causative organism might have been gradually increased to reproduce the disease more completely in later passages. As a matter of fact it was possible to accomplish this in one instance (Case 2).

The injection of blood from some cases of typical yellow fever into guinea pigs caused a rise of temperature in about 5 days similar to that of other cases, and there was even a suspicion of a trace of icterus in the scleras, yet no protection against a later infection with a passage strain of *Leptospira icteroides* (Case 1) could be demonstrated. Perhaps this lack of protection may be explained by assuming the strain variations among different strains of the leptospira.

Another point of interest brought out in this series is that the injection of organ emulsions obtained from fresh postmortem material (Case 27) conferred upon guinea pigs sufficient protection against a subsequent infection to prevent its being fatal, although these animals showed fever and jaundice. It is possible that the emulsion contained a certain amount of the antigen and produced a mild immunity, or a limited amount of the immune bodies.

#### SUMMARY.

The majority of guinea pigs inoculated with the blood of yellow fever patients escaped a fatal infection.

There were a number of instances in which the inoculation of yellow fever blood induced in these animals a temporary febrile reaction on the 4th or 5th day, followed in some cases by slight jaundice, but with a rapid return to normal. Most of these guinea pigs when later inoculated with an organ emulsion of a passage strain of *Leptospira icteroides* resisted the infection. On the other hand, the animals which had previously been inoculated with the blood of malaria patients or normal guinea pigs died of the typical experimental infection after being inoculated with the infectious organ emulsion.

It appears from the results just described that a number of non-fatal, mild, or abortive infections follow the inoculation of blood of yellow fever patients into guinea pigs. The fact that such animals manifested refractoriness to a subsequent attempt to infect with a highly virulent passage strain of *Leptospira icteroides* is an indication, judging from the reciprocal immunity reaction, that they actually passed through an infection with the same organism, or a strain closely related to it, as that which was used for the second infection experiment.

## ETIOLOGY OF YELLOW FEVER.

### V. PROPERTIES OF BLOOD SERUM OF YELLOW FEVER PATIENTS IN RELATION TO *LEPTOSPIRA ICTEROIDES*.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 2, 1919.)

Following the isolation of *Leptospira icteroides* from a case of yellow fever a series of experiments was instituted with a view to establishing the relation between the organism and the disease. As stated elsewhere,<sup>1</sup> the organism reproduces in experimental animals all the symptoms and lesions observed in man. From the standpoint of immunity the question arose whether or not the serum of yellow fever convalescents would have a specific influence upon the organism.

Blood was drawn from the median basilic vein of the patient and the serum mixed with *Leptospira icteroides* in the form of organ emulsion from infected guinea pigs or culture and injected into the peritoneal cavity of a normal guinea pig. After a period of 30 minutes to 1 hour the fluid was drawn from the peritoneal cavity by means of a sterile capillary pipette and examined under the dark-field microscope (Pfeiffer's phenomenon). The animals were allowed to live until the results of the inoculations were evident. As Table I shows, in some of the experiments, in which the amount of the infectious emulsion or culture used was too large, no certain protection of the animal from the final fatal infection was obtained with the serum of convalescents, notwithstanding the fact that such serum produced a definite Pfeiffer phenomenon in the peritoneal cavity. When a smaller quantity of the infecting material was used in combination with convalescent serum most of the animals were saved, while the controls with serum from patients not suffering from

<sup>1</sup> Noguchi, H., *J. Exp. Med.*, 1919, xxix, 585.



Effect of Serum from Yellow Fever Patients upon <i>Leptospira icteroides</i> .										
Case No.	Sex.	Age.	Severity of infection.	Day on which serum was taken.	Amount of serum.		<i>Leptospira icteroides</i> , Strain 1.		Pfeiffer phenomenon	Result.
					cc.	cc.	Liver emulsion.	Culture.		
		yrs.			cc.	cc.	cc.			
9	M.	19	Mild. Recovered.	2nd	1	0.2		—	Died in 8 days.	
9	"	19	" "	10th	1	2		+	Survived.	
2	"	23	" "	12th	2	2		+	"	
14	"	23	Severe. "	10th	2	2		<+	Died in 7 days.	
Control.	"	21	Malaria.		2	2		—	" " 7 "	
"	"	20	"		2	2		—	" " 7 "	
"					Saline, 1 cc.	2		—	" " 7 "	
"					Saline, 2 cc.	2		—	" " 6 "	
7	M.	21	Severe. Recovered.	16th	1	0.1		+	Survived.	
12	F.	40	" "	11th	2	0.1		+	"	
9	M.		Diagnosis (?). Recovered.	30th	2	0.1		—	Died in 6 days.	
Control.					Ringer, 2 cc.	0.1		—	" " 10 "	
"					Ringer, 2 cc.	0.1		—	" " 9 "	
16	M.	25	Severe. Recovered.	19th	1		1	<+	All these sera	
26	"	21	" "	18th	2		1	—	were separated	
29	F.	34	Mild. "	13th	1		1	—	rated from	
19	M.	25	Severe. "	24th	2		1	+	the clot in 48	
30	"	21	Mild. "	14th	2		1	+	hrs. and then	
31	"	22	Moderate. "	20th	1		1	+	kept on ice for	
23	"	16	" "	22nd	2		1	+	2 to 4 wks.	
32	"	20	Mild. "	15th	2		1	+	when they	
33	"	11	" "	7th	2		1	+	were tested.	
8	"	21	Severe. "	14th	2		1	+	The negative	
20	"	20	Moderate. (also malaria).	13th	2		1	<+	results with the sera of Cases 26 and 29 may be due to deterioration through age.	
34	F.	30	Mild. Recovered.	12th	2		1	+		

yellow fever, and with saline solution died with the typical symptoms.

The number of cases studied (eighteen) was limited, owing to the pressure of more urgent problems under investigation at the same time. It seems, however, to have been sufficient to establish the specific reaction which exists between *Leptospira icteroides* and the serum of yellow fever convalescents (fifteen positive, 83 per cent). In the case of one patient the serum did not have any effect upon the organism when tested on the 2nd day of the illness but was protective on the 10th day.

Sera from malarial patients in no case showed any action antagonistic to *Leptospira icteroides*. The malarial patients were all mountaineers and consequently non-immune to yellow fever. The negative results obtained with sera derived from doubtful cases of yellow fever, all so mild as to make it difficult to recognize the disease, might have been due to the absence of antibodies, or, if there were any, to the fact that they were too weak to produce a definite reaction under the experimental conditions, or, a not impossible assumption, to the existence of a variation among many strains.

As will be described later, ten normal sera from healthy, non-immune soldiers<sup>2</sup> were also examined before the vaccination of the latter with killed cultures of *Leptospira icteroides*, but none of them had any effect upon the organism.

#### SUMMARY.

The serum from a number of persons recovering from yellow fever in Guayaquil was studied with a view to establishing its possible immunological relationship with a strain of *Leptospira icteroides* derived from one of the yellow fever patients. For this purpose the serum of convalescents was mixed either with an organ emulsion of a passage strain, or with a culture of the organism, and inoculated intraperitoneally into guinea pigs.

<sup>2</sup> I am indebted for these specimens to Dr. Carlos A. Miño, Assistant Director of the Department of Health at Quito, to Dr. E. Salgado V., also of the Department of Health, and to Dr. Víctor M. Bayas, Surgeon of the "Bolivar" Regiment.

The Pfeiffer reaction was first studied, and then the animals were allowed to live until the controls, inoculated with the same emulsion or culture of *Leptospira icteroides* but without the serum, or with serum from patients suffering from other diseases than yellow fever, had died of the experimental infection with typical symptoms. A positive Pfeiffer phenomenon was observed in fifteen of the eighteen convalescent cases studied, or approximately 83 per cent. Sera from ten non-immune soldiers and from two malaria patients gave uniformly negative results. Protection from an ultimate fatal infection was afforded some of the guinea pigs which received the serum of yellow fever convalescents, while the control animals succumbed to the infection with typical symptoms. In one instance, in which the serum was tested on the 2nd and the 10th days of disease, a Pfeiffer reaction was demonstrated, as well as protective property against the infection, in the specimen from the 10th but not in that from the 2nd day.

From the foregoing observations of immunity reactions it appears highly probable that *Leptospira icteroides* is etiologically related to yellow fever.

## ETIOLOGY OF YELLOW FEVER.

### VI. CULTIVATION, MORPHOLOGY, VIRULENCE, AND BIOLOGICAL PROPERTIES OF *LEPTOSPIRA ICTEROIDES*.

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PLATES 1 TO 3.

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#### *Cultivation.*

As the nature of the causative agent of yellow fever was unknown, it was necessary at the beginning of these experiments to formulate a special method of cultivation. The methods employed have been similar to those recommended<sup>1</sup> for the cultivation of *Leptospira icterohæmorrhagiæ* (Inada and Ido). Instead of the serum and citrate plasma of the rabbit or other animal, serum and plasma from non-immune persons were used during the early stage of the cultivation experiments. The principal medium consisted of a mixture of 1 part of the serum and 3 parts of Ringer solution, used in a combination of the liquid form and a form made semisolid by adding melted neutral agar (0.3 per cent), the liquid half (8 cc.) of the medium being superimposed on the semisolid half (8 cc.) in a tall culture tube such as that used in the cultivation of spirochetes.<sup>2</sup>

The first step in the inoculation of the medium was to mix about 0.5 to 1 cc. of the citrate blood, drawn from the median basilic vein of the patient, with the lower or semisolid portion of the medium, while the latter was still in the fluid state (42°C.), and allow the mixture to solidify by cooling. The serum-Ringer dilution was then poured on the semisolid portion and about 0.5 to 1 cc. more of the same blood introduced. A layer of paraffin oil was finally added to

<sup>1</sup> Noguchi, H., *J. Exp. Med.*, 1918, xxvii, 575.

<sup>2</sup> Noguchi, H., *J. Exp. Med.*, 1912, xvi, 199.



cover the surface of the medium. When making a culture with the citrate blood from a patient no human citrate plasma was added to the liquid portion of the medium, as the plasma contained in the blood was sufficient to form a loose fibrin throughout that portion. When subsequent subcultures were set up, however, 0.5 to 1 cc. of citrate human or rabbit plasma was introduced into the liquid portion after inoculation. The presence of a loose cobweb fibrin in the culture medium seems to favor the growth of certain organisms.<sup>1</sup> The conditions provided for in this form of culture medium would allow the growth of various microorganisms requiring different degrees of oxygen tension. In a later period 2 to 3 cc. of the citrate blood were used for each of two or three large flasks (50 cc.) with correspondingly larger quantities of culture medium (25 cc.).

#### *Direct Cultivation from Yellow Fever Patients.*

Cultures were made from eleven cases of yellow fever, with only three successful isolations of the leptospira. In the first instance (Case 1) I failed to detect the organisms under the dark-field microscope, but in a culture 3 days old (kept at 26°C.) a few active leptospiras were seen. This was inoculated into four guinea pigs, all of whom died later of the typical experimental infection. One of the guinea pigs had epistaxis and melena in addition to intense jaundice, advanced degeneration of the liver, acute parenchymatous nephritis, ecchymoses in the lungs, stomach, and intestines.<sup>3</sup> This culture was presently lost through a secondary fungus contamination, which was difficult to avoid under the conditions in which the work had to be carried on.

The second positive growth was obtained with the blood derived from Case 4 on the 3rd day of the disease. The organisms were readily detected in the culture after 5 days at 30°C. A few leptospiras were present in the blood when carefully examined in the stained preparations, and the guinea pigs inoculated with this specimen came down with the typical symptoms in 8 days. The culture proved to be pathogenic for guinea pigs.

<sup>3</sup> Noguchi, H., *J. Exp. Med.*, 1919, xxix, 565.

The third successful direct cultivation of the organism from yellow fever patients was obtained with the blood from a fatal case (Case 6). The blood was drawn on the 5th day of the disease and put immediately into six tubes containing the culture medium (October 19, 1918). On October 26 one of the inoculated tubes showed the presence of the leptospira under the dark-field microscope. The culture proved to be capable of producing the typical symptoms and lesions in guinea pigs, pups, and marmosets.<sup>4</sup>

*Cultivation from Experimental Animals.*

The method employed for obtaining a culture of *Leptospira icteroides* was the same as that outlined for direct cultivation from human blood, except that normal rabbit serum and citrate plasma were used instead of human. The blood was obtained from the heart before the death of the animal. The method was not always successful in the first generation, but it was nevertheless the most reliable of the various combinations tried. In a later subculture the addition of the citrate plasma becomes unessential, although a better growth is had when it is added.

Six strains (Cases 1 to 6) of yellow fever leptospira have been maintained to date by passage in guinea pigs. No direct culture was obtained with the blood from Cases 2, 3, and 5, although cultures were finally obtained from the blood of guinea pigs or marmosets inoculated with the passage strain. These cultures, whether obtained directly from the blood of yellow fever patients or indirectly by way of animal inoculation, were found to be uniform in their characteristics and could be maintained in culture for many months. The leptospira isolated from yellow fever cases is extremely sensitive to any alien microbic intrusion, not surviving the slightest contamination in culture, and the failure to obtain a culture directly from yellow fever cases can in every instance be explained through the occurrence of secondary contamination.

When the guinea pigs reach the stage of collapse, with intense jaundice, it is seldom possible to detect the leptospira in the circulation, and a positive transfer to normal guinea pigs becomes uncertain.

<sup>4</sup> Noguchi, H., *J. Exp. Med.*, 1919, xxix, 585.

Cultures set up with this blood usually remain sterile, and the leptospira is not found in the liver and kidneys. In experimental infectious jaundice, on the contrary, the leptospira was almost always found in the later stage of the infection. The extreme lability of *Leptospira icteroides* may account for the negative animal inoculation and microscopic findings in so many cases of yellow fever.

### *Morphology.*

The organism which occurs in the blood and tissues in yellow fever patients in Guayaquil, as well as in those of animals experimentally infected with the blood or tissue of yellow fever patients, is an extremely delicate filament measuring about 4 to 9 microns in length and 0.2 of a micron in width along the middle portion. It tapers gradually toward the extremities, which end in immeasurably thin sharp points. The entire filament is not smooth but is minutely wound at short and regular intervals, the length of each section measuring about 0.25 of a micron. The windings are so placed as to form a zigzag line by the alternate change of direction of each consecutive portion at an angle of 90°.

The organism is unrecognizable by translucent light but becomes quite visible under a properly adjusted dark-field illumination. It possesses an active motility, consisting in vibration, rotation, rapid bipolar progression, and sometimes twisting of parts of the filament. When it encounters a semisolid substance it penetrates the latter by a boring motion, and while passing through it the body assumes a serpentine aspect with few undulations, the elementary windings undergoing no modification.

The organism manifests remarkable flexibility to almost any angle while changing its course of progression in a semisolid medium. In a fluid medium it has fewer and quite characteristic movements. One end is usually bent in the form of a graceful hook, and, while rapidly rotating, the organism proceeds in the direction of the straight end, the hooked end apparently serving as a sort of rear propeller. When extricating itself from an entanglement, however, the same hooked end seems to act like the front propeller of an airplane. Many specimens are seen with both ends hooked, the organism then ro-



tating in a stationary position unless one hook is larger and more powerful as a propeller than the other. The rapid rotation makes the organism appear like a chain of minute dots. From the dynamic point of view the portions which include the several windings from the extremities represent the motor apparatus of the organism. I have never seen a specimen that doubled at the middle portion of the body while lying in a free liquid medium. The motor or terminal portions may be regarded as comparable with the flagella or terminal filaments seen in a spironema or treponema.

The organism is difficult to stain with ordinary aniline dyes, but can be made distinct by osmic acid fixation and one of the Romanowsky stains (Giemsa, Wright, Leishman). When stained with Fontana or carbolized gentian violet solution after mordanting with 5 per cent tannin plus 1 per cent phenol the organism appears as a moderately heavy, slightly undulated filament without a clear elementary indentation. The peculiar forms resembling the letters C and S are quite characteristic. Specimens fixed with methyl alcohol seldom retain the elementary spirals. The beauty of the organism as it appears by dark-field illumination is never well retained in a stained preparation, even in the best specimens. In the latter it appears almost as a totally different organism.

From the findings described it is evident that the present organism falls in the general order of so called spirochetes, but in the strict sense of the term it is neither a bacterium, a spirochete, a spironema, nor a treponema, but belongs to the genus *Leptospira*, of which *Leptospira icterohæmorrhagiæ*, *Leptospira hebdomadis*,<sup>5</sup> and *Leptospira biflexa* have already been described.<sup>1</sup>

The study of the strains of *Leptospira icteroides* obtained from yellow fever cases in Guayaquil showed the organisms to be of somewhat smaller dimensions than the various strains of *Leptospira icterohæmorrhagiæ* in my possession (six strains), as is readily seen from the photographs of the organisms shown in Figs. 1 to 9. The difference is striking when the pictures of the two organisms are compared, particularly in the case of Strain 6 of *Leptospira icteroides*, which is considerably smaller than any of the other strains.

<sup>5</sup> Ido, Y., Ito, H., and Wani, H., *J. Exp. Med.*, 1918, xxviii, 435.



The strains of *Leptospira icterohæmorrhagiæ* isolated from wild rats caught in Guayaquil are also shown (Figs. 10 to 13). These are seen to be similar to the other strains of *Leptospira icterohæmorrhagiæ*, shown in Figs. 5 to 9, and are coarser than the organism obtained from yellow fever cases.

The photographs shown in the three plates were taken for the purpose of comparison at the same time, under similar conditions, and with the same magnification.

### *Cultural Properties.*

*Leptospira icteroides* does not multiply in a medium in which there is no access to oxygen. In a dense solid medium it grows well within the zone or layer to which a trace of oxygen can still penetrate, but no deeper. It grows best when the supply of oxygen is not excessive, as when a thin layer of liquid paraffin is poured over the surface of the culture medium. A certain amount (above 10 per cent) of a suitable blood serum is essential for its growth. Various bacterial culture substances such as peptone, meat extract, various carbohydrates in different forms, or combinations, are unsuitable; their presence in the serum-containing media apparently neither favors nor impedes development. The percentage of sodium chloride (tried as high as 2 per cent) in the medium seems to have but little influence, and either isotonic saline, Ringer solution, or distilled water may be used as diluent. The organism is highly sensitive to the reaction of the medium, the optimum growth being obtained with a reaction slightly alkaline to litmus paper, not stronger than 0.025 N. It grows well in a neutral medium, but not in one with an acid reaction to litmus paper.

The addition of phenol red to culture media in a ratio of 1 cc. of a 0.0025 per cent solution to 10 cc. of medium has no perceptible disturbing effect upon the growth of *Leptospira icteroides*. Growth takes place in culture tubes in which phenol red indicates the values ranging from pH 6 to pH 7.4. In the case of cultures containing rabbit serum phenol red is gradually decolorized to a trace of pink.

Growth is much more rapid at a temperature of 37°C. than at 25–26°C., but the organisms remain viable much longer at the latter

temperature. No growth is obtainable at a temperature above 42°C. or below 10°C.

Erythrocytes present in the culture do not undergo any special alteration that can be ascribed to the growth of this organism, nor does the hemoglobin. The serum proteins seem in no wise modified and remain transparent. No external changes of the culture media, except a light layer of grayish haze over the surface of a solid or semisolid medium observed in richly growing old cultures, take place, and for this reason the growth of the organism can be ascertained microscopically only.

It has been noticed that *Leptospira icteroides* shows a particular preference for a semisolid medium such as is provided by the presence of agar (0.3 per cent) or by loose fibrin. They entangle themselves in the substance in large numbers and move about in it very actively. Continuous multiplication goes on in this type of medium. After a few weeks the growth may become so dense as to render the uppermost layer of the medium faintly grayish. This peculiarity of the organism may partly account for its predilection for the parenchymatous organs such as the liver and kidney.

*Leptospira icteroides* multiplies through transverse fission.

#### *Virulence.*

While the pathogenic properties of *Leptospira icteroides* for different species of animals have not been exhaustively studied, it has been shown that most of the domestic animals, such as the donkey, horse, sheep, pig, and cat are completely refractory to the injection of the organism. Very young dogs, not older than 6 or 7 weeks, are found to succumb to experimental infection. None of the birds so far employed for experiment has been found to be susceptible.<sup>3</sup> Among the mammals the guinea pig appears to be most susceptible and the marmoset somewhat less so. For this reason the guinea pig has been chosen for determining the degree of virulence of several strains of *Leptospira icteroides*.

The mode of inoculation consisted in intraperitoneal injection in descending doses of a culture 2 to 3 weeks old. The guinea pigs used varied from 300 to 350 gm. A 2 to 3 weeks old culture (26°C.) of this organism, grown in a semisolid rabbit serum medium with

0.15 per cent agar, may contain 50 to 100 leptospiras per field (Leitz  $\frac{1}{2}$  oil immersion and ocular 4). In order to arrive at an accurate determination of virulence different strains would have to be used in correspondingly comparable concentrations. But this is extremely difficult in the case of an organism which forms entangled masses of many individuals or shows a tendency to congregate in varying numbers about the particles of culture medium. In the present series of experiments suspensions of cultures of different strains were so prepared as to make each contain approximately an equal number of organisms in the suspension from which higher dilutions were prepared. Each strain was used in successive tenfold dilutions, and one or two guinea pigs were inoculated with 1 cc. each of each dilution. Because

TABLE I.  
*Determination of Virulence of Leptospira icteroides, Strain 1.*

Guinea pig No.	Quantity of culture.	Incubation, or time after inoculation to onset of fever.	Result.
	cc.	days	
1	1	3	Died in 7 days.
2	0.1	4	Survived (!). No jaundice.
3	0.01	5	Died in 9 days.
4	0.001	3 $\frac{1}{2}$	" " 8 "
5	0.0001	4	" " 9 "
6	0.00001	5	" " 10 "
7	0.000001	No fever.	Survived.

of individual variations in resistance among the guinea pigs in all later experiments two animals were used for each dilution.

Four strains of *Leptospira icteroides* were studied in this way. The results are recorded in the following protocols.

*Experiment 1.*—Aug. 10, 1918 (at the Guayaquil Yellow Fever Hospital). Strain 1. 18 day culture of the second generation, grown on semisolid human serum agar at 30°C. (Table I.)

*Experiment 2.*—Dec. 2, 1918. Strain 3. 20 day culture of the third generation, grown on semisolid rabbit serum agar medium at 26°C. (Table II.)

*Experiment 3.*—Jan. 2, 1919. Strain 5. 3 week culture of the third generation, isolated from a marmoset experimentally infected with a visceral emulsion from a guinea pig which died of the typical infection after inoculation with blood from a fatal yellow fever case. The culture was grown on the same medium as that used for Strain 6, at 26°C. (Table III.)

TABLE II.  
*Determination of Virulence of Leptospira icteroides, Strain 3.*

Guinea pig No.	Quantity of culture.	Incubation, or time after inoculation to onset of fever.	Result.
	cc.	days	
1	1	3½	Died in 8 days.
2	1	5	Survived (!).
3	0.1	4	Died in 9 days.
4	0.1	5	" " 8½ "
5	0.01	5	Survived (!).
6	0.01	5½	Died in 11 days.
7	0.001	4	" " 9 "
8	0.001	5	Survived.
9	0.0001	5	"
10	0.0001	No fever.	"
11	0.00001	7	"
12	0.00001	No fever.	"
13	0.000001	" "	"

TABLE III.  
*Determination of Virulence of Leptospira icteroides, Strain 5.*

Guinea pig No.	Quantity of culture.	Incubation, or time after inoculation to onset of fever.	Result.
	cc.	days	
1	1	3	Died in 7 days.
2	0.1	3	" " 8 "
3	0.1	2½	" " 6 "
4	0.01	3½	" " 7 "
5	0.01	4½	" " 9 "
6	0.001	5	" " 10 "
7	0.001	3½	" " 8 "
8	0.0001	5	" " 10 "
9	0.0001	4	" " 9 "
10	0.00001	6	Survived.
11	0.00001	No fever.	"
12	0.000001	" "	"
13	0.000001	" "	"

*Experiment 4.*—Jan. 2, 1919. Strain 6. 20 day culture of the third generation, directly derived from human blood (not passed through guinea pigs), grown on semisolid rabbit serum agar at 26°C. There were about 25 organisms per field in the original suspension. (Table IV.)



The foregoing experiments show that the strains of *Leptospira icteroides* possess, on the whole, a strong virulence for guinea pigs. In two cases (Nos. 1 and 6) the minimal lethal dose was 0.00001 cc., in one (No. 5) 0.0001 cc., and in another (No. 3) 0.001 cc. But in the experiments with Strains 1 and 3 some of the guinea pigs receiving as large a quantity as 1 cc. or 0.1 cc., showed only a transient febrile reaction and speedily returned to normal, notwithstanding the fact that 0.0001 part of these doses killed other guinea pigs in the same series of experiments. This is not altogether exceptional,

TABLE IV. .

*Determination of Virulence of Leptospira icteroides, Strain 6.*

Guinea pig No.	Quantity of culture.	Incubation, or time after inoculation to onset of fever.	Result.
	cc.	days	
1	1	4	Died in 7 days.
2	0.1	3	" " 10 "
3	0.1	3½	" " 6 "
4	0.01	4	" " 8 "
5	0.01	5 (?)	" " 7 "
6	0.001	3	" " 9½ "
7	0.001	5	" " 10 "
8	0.0001	4	" " 10 "
9	0.0001	Doubtful.	Survived.
10	0.00001	3	Died in 8 days.
11	0.00001	6	Survived.
12	0.000001	4	"
13	0.000001	No fever.	"

because in the higher dilutions there were instances in which a smaller dose induced a fatal infection while a larger one failed to do so. Again, in fatal instances the severity of the infection did not parallel the amount of culture injected. In other words, the susceptibility of guinea pig varies considerably among different individuals. In another series of experiments, not yet reported, it was noticed that certain guinea pigs possess an almost complete natural immunity to *Leptospira icteroides*. This becomes an important factor in a consideration of the percentage of successful transmissions of this organism from human cases to guinea pigs.

*Gradual Loss of Virulence through Cultivation.*

All the strains of *Leptospira icteroides* were brought to New York from Guayaquil on semisolid rabbit serum agar. The cultures were kept at ordinary temperature during the journey (about 28°C. in the tropics and 15°C. after reaching the United States). They had been renewed in Guayaquil on October 26, 1918, and were tested for their pathogenicity for guinea pigs in New York on December 2, 1918; that is, 37 days after the transfer into new media. Intraperitoneal inoculations were made into guinea pigs of 1 cc. of Strains 1, 3, 4, 5, and 6, and the animals developed the usual symptoms and lesions characteristic of the infection produced by these strains, showing that under the circumstances described the organism remained virulent for 37 days.

On December 9, 1918, some of the older cultures of Strains 1 and 3, which had stood over 4 months since cultivation, were also tested, with varying success. Strain 1 was still quite virulent, but Strain 3 failed to produce a fatal infection. In a subsequent experiment, however, by using six guinea pigs, each being inoculated with 2 cc. of the culture intraperitoneally, it was possible to obtain a fatal infection in one of the animals. Through this guinea pig the virulence of the culture was again raised to its original height; that is, it again became capable of causing typical infection in guinea pigs in smaller quantities.

It should be noted that examination of the viscera, especially the lungs, of the guinea pigs which escaped death or severe infection from the inoculation of an attenuated strain, by killing them at the end of about 14 days from the time of inoculation, usually revealed the presence of old hemorrhages of greater or less extent in the lungs. Perhaps it may prove a useful procedure for ascertaining the outcome of transmission to inoculate several guinea pigs with the blood of a yellow fever patient and examine the lungs within a period of from 10 to 14 days. In this way, notwithstanding the absence of striking external manifestations, the results of inoculation can be more accurately followed. Unfortunately this fact was not known at the time of the experiments, reliance being placed upon the development of a fatal infection.

For the past 4 months horse serum has been used for culture media, since it was easily obtainable at much less expense and in larger quantities than rabbit or sheep serum. It is far less satisfactory, however, for the cultivation of *Leptospira icteroides*, than sheep serum, which in turn is much inferior to rabbit serum. In testing out the virulence of different strains of the organism recently, rather rapid loss of virulence has been encountered. The reason was not at first clear, the period of time being comparatively short, but it soon became evident that cultivation of *Leptospira icteroides* on sheep or horse serum media leads to rapid diminution of virulence, since the cultures grown on rabbit serum media remained pathogenic. By following the course of development of these strains in media containing rabbit serum and phenol red it was found that the original pinkish color of the indicator gradually becomes paler until it fades to a trace. The color may be brought back to a deeper hue by the addition of disodium phosphates, but it never reaches the original grade. Apparently a change takes place in the media as well as in the indicator through the growth of the organism. Such a change has not been observed in the cultures grown on media containing sheep or horse serum. Whether the persistence of virulence of the organism in the media containing the rabbit serum has any relation to the phenomenon just described or whether they are two unrelated coincidental phenomena has not been further studied.

#### *Resistance and Viability.*

*Leptospira icteroides* is a non-spore-bearing organism and offers little resistance to the action of heat, desiccation, putrefaction, or disinfectants.

Heating to 55°C. for 10 minutes or freezing and thawing kill the organism, and complete desiccation promptly destroys its vitality.

In the presence of various bacteria, such as *Bacillus coli*, *Bacillus aerogenes*, *Bacillus subtilis*, *Bacillus mesentericus*, *Bacillus pyocyaneus*, pneumococcus, staphylococcus, *Streptococcus hæmolyticus*, etc., *Leptospira icteroides* is destroyed within a short time. The more numerous the bacteria the quicker the disappearance of the leptospira; hence in decomposing excreta or urine, sewer or stagnant water, or in con-



taminated foodstuffs, no leptospira can be found 24 hours after being introduced into them.

On the other hand, certain contaminating fungi or non-putrefactive and non-acid-producing bacteria, sometimes bacilli and sometimes cocci, have been found growing in the cultures of *Leptospira icteroides* without seriously interfering with the viability of the latter, which are actively motile among the intruding fungi or bacteria. In such a contaminated culture these intruders do not cause any perceptible modification of the culture medium, except that their discrete colonies may be found imbedded here and there in the medium.

When a pure culture of the organism was poured into a cup of sterile distilled water and left unprotected from the air or dust the leptospiras survived several days, but finally disappeared, partly because of the lack of nutrition and partly because of bacterial growth. The leptospira intentionally added in large quantity to fecal matter kept at room temperature disappeared within a few hours.

On several occasions attempts were made to infect the larvæ of *Stegomyia calopus* by introducing emulsions of liver or kidney containing a large number of the organisms into the receptacle with the larvæ, but no leptospira could be found in such a mixture after 2 hours. In this respect the virus of yellow fever is one of the least resistant of all pathogenic organisms which have been obtained in culture. In my experience there has seldom been an impure culture of the organism.

Another interesting phenomenon in connection with the organism in question is that it soon dies out; it may degenerate within 12 hours in a piece of liver or kidney removed from an infected guinea pig and kept at a temperature of about 10°C. In hundreds of instances a leptospira was found only rarely in the liver, kidney, or blood from guinea pigs which had died of typical experimental yellow fever several hours before autopsy. In this respect *Leptospira icteroides* differs considerably from *Leptospira icterohæmorrhagiæ* isolated from the cases of infectious jaundice in Japan or Europe, the latter being still easily recoverable from animals kept over night after death.

With regard to the resistance of the organism to the action of various ordinary disinfectants the work is still incomplete. It has been found, however, that it is readily killed within 5 minutes by 2 per



cent phenol or 0.1 per cent bichloride of mercury. In a 10 per cent solution of sodium taurocholate, sodium glycocholate, or sodium cholate the organism promptly disintegrates, but saponin has no injurious effect upon it. Human or animal bile dissolves the organism rapidly when used in concentrations stronger than 30 per cent.

### *Filterability.*

A noteworthy characteristic of *Leptospira icteroides* is its ability to pass through the pores of filters. Some experiments to determine this point were carried out as early in the investigation as the transmission of Strain 1 from the human case to guinea pigs. By the use of Berkefeld filters V and N with suction by means of a water pump it was possible to filter an emulsion of the liver and kidney of a guinea pig experimentally infected with the passage strain 7 days previously. The clear filtrates, which were bacteriologically sterile, were inoculated intraperitoneally into normal guinea pigs in doses of 10 cc. each on August 8, 1918. Both animals came down with typical symptoms after  $7\frac{1}{2}$  and 8 days respectively. In the blood of these animals a small number of leptospiras were demonstrated 24 hours before death. In the emulsions of the liver and kidney the organisms were also present, and upon further passage to normal guinea pigs the emulsion proved to be infectious.

### *Possibility of the Existence of a Granular Phase in the Life of Leptospira icteroides.*

That there may exist a granular phase of life in various members of the family of spirochetes has been repeatedly suggested by investigators. Balfour, Fantham, Leishman, and Todd<sup>6</sup> advanced the idea that the spirochetes of relapsing fevers in man and fowls pass through a granular stage at some time in their life. The following observation seems strongly to suggest the possibility that this phenomenon also occurs in the life of *Leptospira icteroides*.

<sup>6</sup> Balfour, A., *Internat. Congr. Med.*, 1913, xxi, 275. Fantham, H. B., *Ann. Trop. Med. and Parasit.*, 1914, viii, 471. Leishman, W. B., *Internat. Congr. Med.*, 1913, xxi, 282. Todd, J. L., personal communication.

The various cultures of *Leptospira icteroides* made at Guayaquil on October 26, 1918, were brought back to New York on November 24 without special accident. But on examination no leptospira could be found in any of the tubes containing cultures of Strain 5, although the other cultures were growing well. A thorough examination of the eleven tubes of Strain 5 was continued for several days without success.

Six culture tubes which were made on October 18, 1918, with the blood from Marmoset 4, severely infected with Strain 5, and which had been showing a fairly good growth on October 26, were also examined. These tubes showed no spiral organisms. There were large numbers of refringent granules imbedded in the culture medium in which the leptospiras had been abundantly present a month previously. These granules appeared to be the degenerated remains of the leptospiras. The hope of recovering the strain from these cultures was almost abandoned, but as a last resort a dozen guinea pigs were inoculated with 1 cc. of the contents of each of these tubes. Some of these animals in due time came down with typical symptoms. The spiral forms of the leptospiras were found in varying numbers in the blood, liver, and kidneys of these animals, and a culture of the strain was regained.

It is of course possible that these old culture tubes contained the spiral leptospira in such small numbers that they escaped microscopic detection, but it is also possible that they existed in a granular phase under certain conditions.

#### SUMMARY.

By the employment of methods designed to promote the growth both of aerobic and anaerobic organisms, particularly those belonging to the class of spirochetes, it was possible to obtain a pure culture of a delicate organism, the morphological features of which place it in the genus *Leptospira*. On three occasions, that is, from three out of eleven cases of yellow fever, the organism was directly cultivated. These three strains were found to induce the characteristic symptoms and lesions when tested on guinea pigs. The organism was designated *Leptospira icteroides*.

*Leptospira icteroides* was also obtained in pure culture from the blood of guinea pigs which succumbed to infection after being inoculated with the blood or organ emulsions from patients suffering from yellow fever. These cultures also proved to be virulent when tested on susceptible animals.

The morphological characteristics and certain biological properties of the organism were considered in detail. It is invisible under translucent illumination and is difficult to stain by most aniline dyes. It is highly sensitive to the presence of bacteria and is rapidly destroyed in a medium in which certain other organisms are present. The presence of blood serum (man, sheep, horse, rabbit, etc.) seems to be essential for its growth. It grows well at a temperature of about 25–26°C. and more quickly at 37°C., though at the latter temperature it dies out within a few weeks. At 25°C. under favorable conditions and in suitable culture media it remains viable for several months without losing its virulence. *Leptospira icteroides* multiplies by transverse division.

The virulence attained by some strains was such that 0.00001 cc. of a culture could induce typical fatal infection in guinea pigs. There exists a considerable variation among guinea pigs in their susceptibility to *Leptospira icteroides*.

The organism is killed within 10 minutes at a temperature of 55°C. and is also destroyed by complete desiccation or freezing and thawing. Bile and bile salts dissolve it in certain concentrations, but not saponin.

*Leptospira icteroides* passes through the pores of Berkefeld filters V and N, and there is a possibility of its having a granular phase of life under certain conditions.

#### EXPLANATION OF PLATES.

##### PLATE 1.

FIG. 1. Dark-field view of a 2 week culture on semisolid rabbit serum agar medium of *Leptospira icteroides*. Strain 6 (Case 6).  $\times 3,000$ .

FIG. 2. The same. Strain 4 (Case 4).

FIG. 3. The same. Strain 5 (Case 5).

FIG. 4. The same. Strain 3 (Case 3).

## PLATE 2.

FIG. 5. Dark-field view of a 2 week culture on semisolid rabbit serum agar medium of *Leptospira icterohæmorrhagiæ*. Japanese strain.  $\times 3,000$ .

FIG. 6. The same. British strain.

FIG. 7. The same. French strain.

FIG. 8. The same. American Strain 1.

FIG. 9. The same. American Strain 2.

## PLATE 3.

FIG. 10. Dark-field view of a 2 week culture on semisolid rabbit serum agar medium of *Leptospira icterchæmorrhagiæ*. Group 8 strain obtained from wild rats in Guayaquil.  $\times 3,000$ .

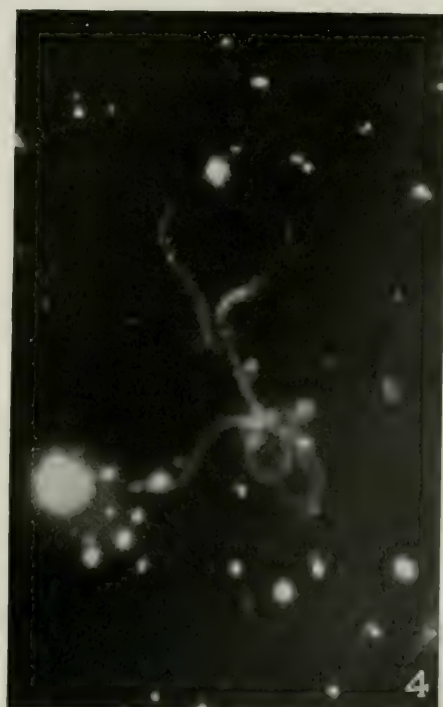
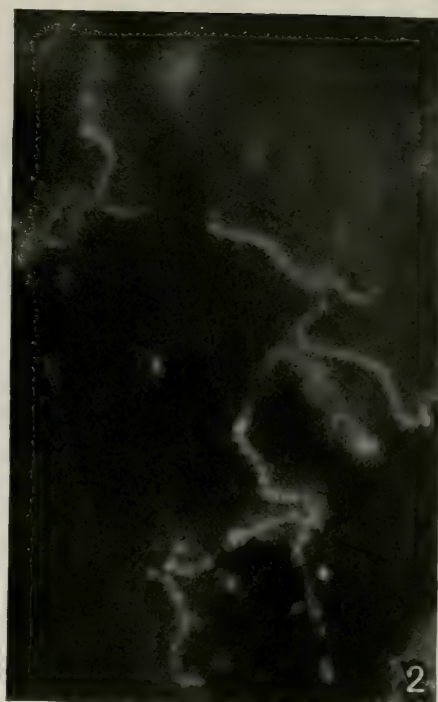
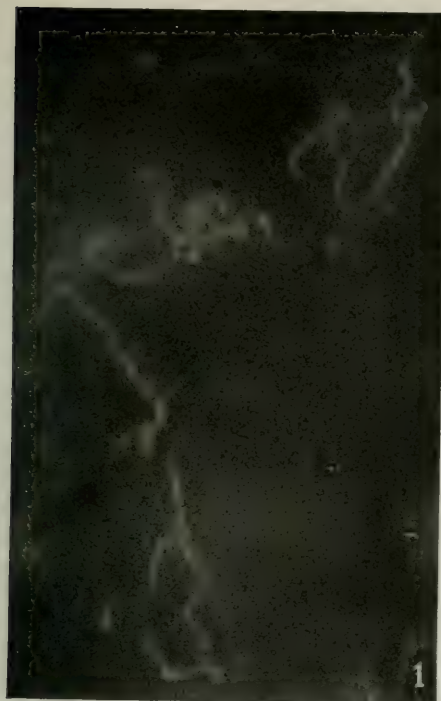
FIG. 11. The same. Group 11 strain.

FIG. 12. The same. Group 30 strain.

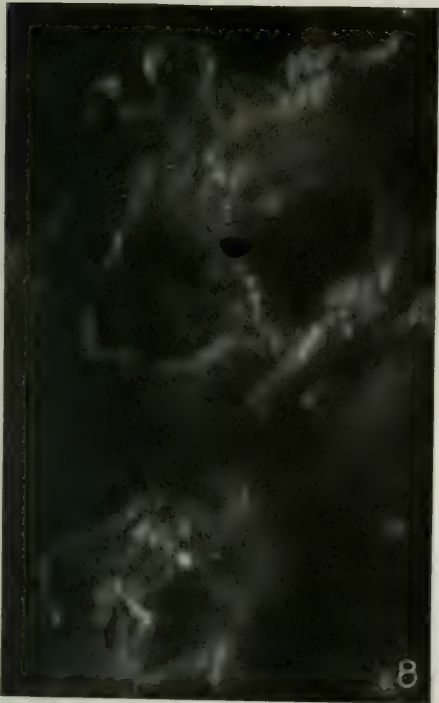
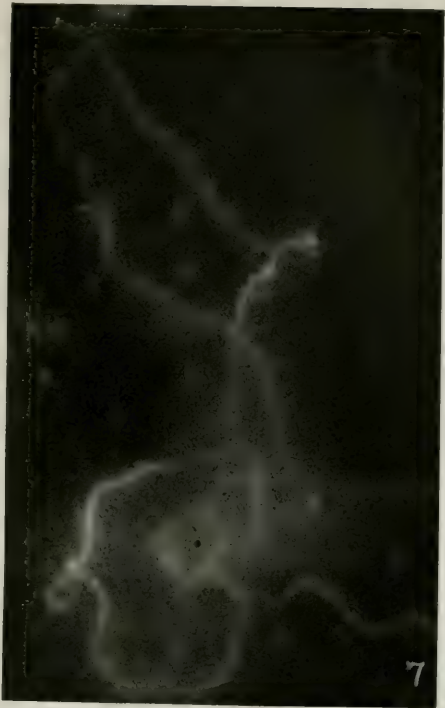
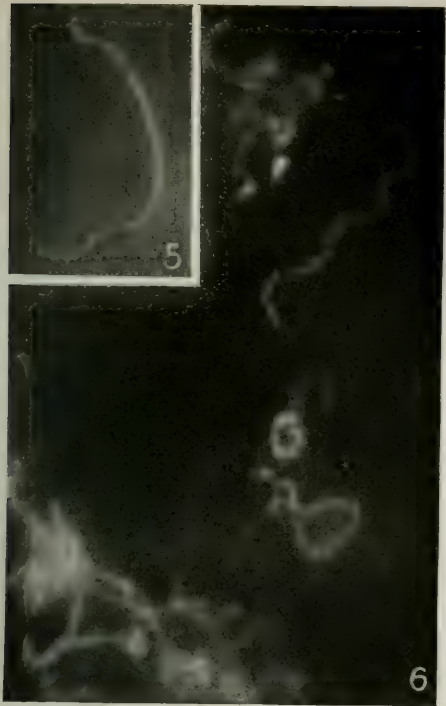
FIG. 13. The same. Group 30 strain.







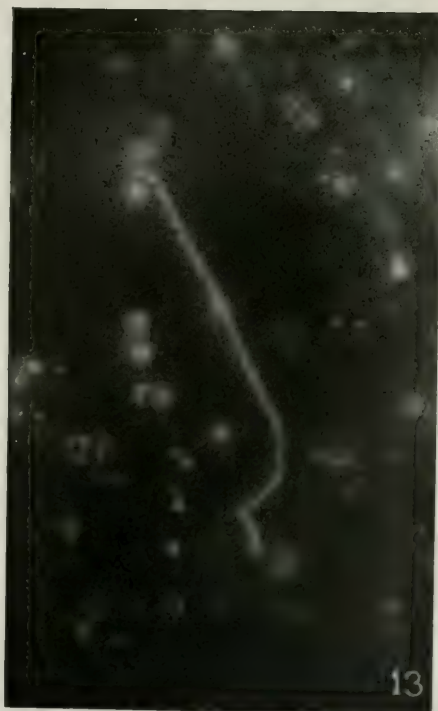
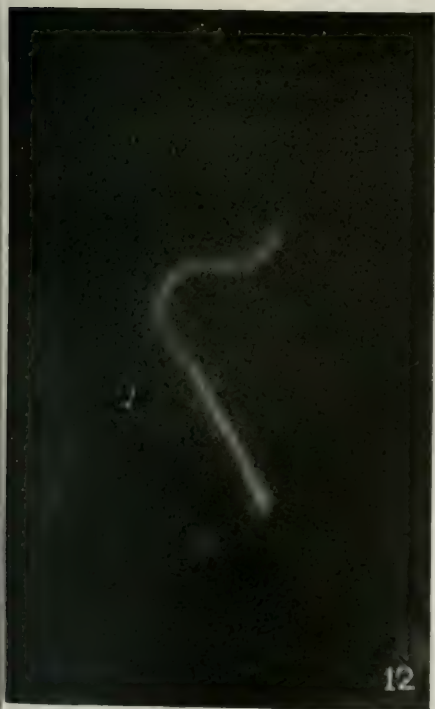
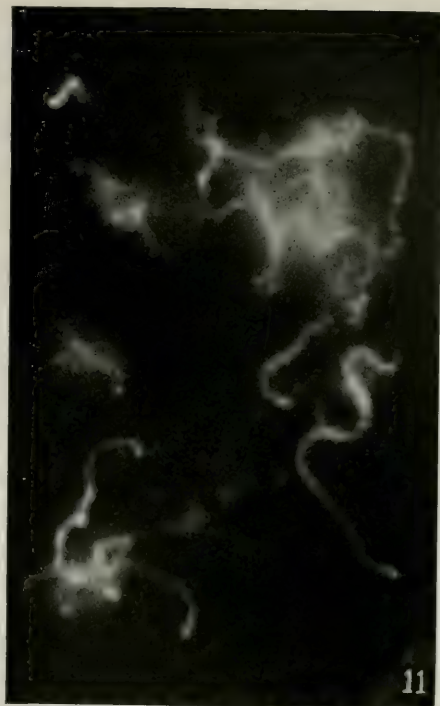
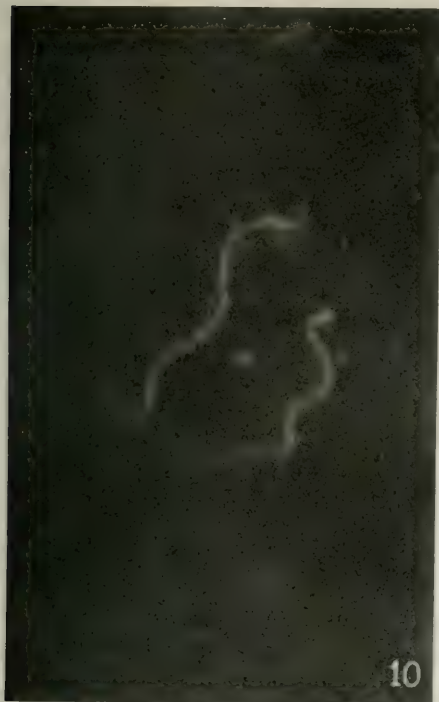








106<sup>3</sup>





## ETIOLOGY OF YELLOW FEVER.

### VII. DEMONSTRATION OF LEPTOSPIRA ICTEROIDES IN THE BLOOD, TISSUES, AND URINE OF YELLOW FEVER PATIENTS AND OF ANIMALS EXPERIMENTALLY INFECTED WITH THE ORGANISM.

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(Received for publication, May 16, 1919.)

The number of *Leptospira icteroides* found in the blood of yellow fever patients was so small that a prolonged examination of blood specimens was necessary to discover one organism, even when positive transmission had been obtained by injection of the blood into guinea pigs. In most cases the organism could not be detected in the blood, perhaps owing to lack of time. To devote much time to microscopic examination during the period while the work with yellow fever patients was being carried on was impossible and also inadvisable, since it was a matter which could be taken up at a later date when more time was available. Hence, what will be reported in this paper is not final but is the preliminary account of what was accomplished under the circumstances. The demonstration of the organism in the blood and various organs of animals experimentally infected with *Leptospira icteroides*, on the other hand, was much more satisfactorily accomplished. The infection could be produced at the desired time and the material obtained in sufficient quantity at any stage of the disease.

#### *Examination of Material from Yellow Fever Cases.*

As already stated<sup>1</sup> a minute organism belonging to the genus *Leptospira* has been found both in the blood and in the tissues of various organs, particularly the liver and kidneys, of guinea pigs

<sup>1</sup> Noguchi, H., *J. Exp. Med.*, 1919, xxix, 565.



which contracted an experimental infection after being inoculated with the blood of yellow fever patients taken during the early stages of the disease. In three instances (Cases 1, 4, and 6) the organism was found in the cultures made directly from the blood of yellow fever patients. A special effort was made, therefore, whenever a case of yellow fever was admitted to the hospital or an autopsy was performed, to find the same organism in the blood and tissues, and if possible also in the urine.

The dark-field microscope was used for examination of the fresh material. Films and sections were stained by Giemsa's or Wright's stain, by Levaditi's or Noguchi's silver impregnation methods, as occasion demanded. Fontana's silver impregnation method was applied several times without satisfactory results. Blood films were usually fixed with methyl alcohol,<sup>2</sup> while the impression films of various tissues from autopsies were first fixed over osmic acid vapor for 1 minute and then in absolute alcohol for 30 minutes. They were stained with Wright's stain for 30 minutes and then with Giemsa's stain over night. Six to twelve were prepared from each specimen of blood or tissue for examination.

Of twenty-seven cases of yellow fever in only three (Cases 3, 4, and 14) was the leptospira demonstrated in the blood under the dark-field microscope, and in each instance the number of the organisms found was so small that once they had passed out of the field they were not easily encountered again. The blood of two of these three patients (Cases 3 and 4) also yielded positive transmission into guinea pigs.<sup>1</sup> The organism was found in a few of the stained film preparations, but these will have to be repeatedly examined. Some specimens of yellow fever blood, however, were infectious for guinea pigs even when in the fresh state no leptospira could be demonstrated under the dark-field microscope (Cases 1, 2, 5, and 6). That there must have been a very small number of the organisms in such specimens is shown by the fact that the blood and organs of the infected animals contained the organisms. A negative microscopic examination of the blood indicates either absence or scarcity of the organism. Nor does the failure of the blood to reproduce a fatal experimental

<sup>2</sup> For the purifying of the methyl alcohol for this purpose I am indebted to Dr. Herman Edward Redenbaugh of the Commission.

infection in guinea pigs prove the absence of the organism in the specimen, since variations in its pathogenicity for guinea pigs are considerable (Case 14).

Examination of various organs under the dark-field microscope has yielded so far only one positive finding, that in the liver of Patient J. Co. (Case 4), who died on the 4th day of the disease. The liver was excised from the body (a partial autopsy) a few hours after death while the body was still quite warm. The kidney failed to show any leptospira. In the stained specimens of the blood, liver, and kidney a small number of leptospiras was demonstrated. Both the blood and the liver emulsion of this patient yielded a positive transmission of the disease to guinea pigs.<sup>1</sup>

A careful search for the organism was carried out with the films made from the liver, kidney, lungs, adrenals, mesentery, and inguinal glands from ten more cases, with so far a positive finding in the liver of one case (Case 5). Some of these slides were poorly stained and will have to be repeatedly examined when more time is available. In a later paper will be recorded the results of the examination of sections of the organs from patients who died of yellow fever.

Specimens of urine from twenty-one cases of yellow fever were examined under the dark-field microscope, but no leptospira was encountered. The examinations were made during the height of the illness, which is usually the 5th or 6th day of the disease, as well as during convalescence, towards the end of the 2nd week. This part of the investigation was much handicapped by the lack of a powerful centrifuge to concentrate the urine, and it will have to be repeated under more favorable conditions. As might be expected, *Treponema minutum*<sup>3</sup> and *Treponema calligyrum*<sup>4</sup> were occasionally seen in the samples of urine. A doubtful result was obtained in a guinea pig inoculated with 10 cc. of the urine from a convalescent (Case 51) on the 15th day of the disease, although no leptospira was found in the specimen.

<sup>3</sup> Noguchi, H., *J. Exp. Med.*, 1918, xxvii, 667.

<sup>4</sup> Noguchi, H., *J. Exp. Med.*, 1913, xvii, 89.

*Examination of Material from Experimentally Infected Animals.*

Experiments were undertaken to determine the approximate time when the blood, liver, and kidney of guinea pigs infected with the yellow fever virus first contain enough organisms to produce the infection when transferred to a normal guinea pig, and when they cease to be infective. These points are more of academic interest, as they do not affect the present sanitary measures with regard to isolation of the patients before, during, and after the actual sickness.

When a minute quantity of the virus is introduced into the subcutaneous tissue of the guinea pig no local reaction follows. The blood becomes infective as early as 48 hours after the inoculation in some instances, but more constantly so after 72 hours. The liver and kidney become infective simultaneously with the blood. The amount of blood effective for infection is comparatively large, at least 1 cc. being required during the earlier period. The demonstration of the organism in the blood is almost hopeless and has not been successful until the 5th day after inoculation, when occasional specimens have been found after a long search. The organisms are more readily found in the emulsions of the liver or kidney, sometimes more numerous in one and sometimes in the other. After the onset of the disease the number of organisms gradually increases for a time, both in the blood and in the liver and kidney. Then the relation of the organism to the blood on the one hand and to the liver and kidney on the other seems to show a certain difference. The organisms continue to increase in the organs somewhat longer than in the blood. In fact, the number of organisms in the blood becomes fewer as the disease progresses, and only a few can be detected when the jaundice and other symptoms have fully developed; that is, a day or two before death. This has been the rule with the majority of the guinea pigs in the present experiments, but there were exceptions in which the organisms were quite numerous until death. Although they increase in the liver and kidney in greater number and for a longer period, they have been found in most instances finally to disappear also from these organs. The disappearance is sudden and usually occurs about 24 hours before death, although there are instances in which they can be found in large numbers at death, sometimes



more abundantly in one or the other organ. Although it is true that when the blood contains the leptospira at the time of death the liver and kidney also contain it, yet the reverse is not always the case, the organism being found in the organs but not in the blood. An instance has not been met in which the leptospira was present in the blood but not in the liver or kidney or both.

The mechanism of the disappearance of the leptospira in so many typical experimental guinea pigs during the later stage of the infection is difficult to explain, but it may be partly due to the formation of certain still undetermined metabolic products brought about by the disorganization of the liver or kidney or some other organs. The leptospira is highly sensitive to the dissolving action of bile salts *in vitro*, and it is not inconceivable that a predecessor or a derivative of taurocholic, glycocholic, or cholic radicals in a certain stage may exert a powerful destructive action upon it.

With regard to the question of the infectivity of the blood and organs in the later stage of the infection, the statement may be made that in all instances in which the organism was seen, whether in the blood or in the organs, the material was always infective. On the other hand, in a considerable number of instances in which the dark-field search for the organism failed, no infection could be induced in normal guinea pigs by inoculation of the material. It may be supposed that the organisms had completely disappeared from the body in these cases. However, as was to have been expected, there were many instances in which, notwithstanding an unsuccessful microscopic search for the organism, inoculation of the liver or kidney, or both, but not the blood, gave rise to a typical infection in further passage, with the reappearance of the leptospira in the new host. In one instance the leptospira was microscopically detected on the third passage. It may be concluded, then, that in experimental infection in the guinea pig the specific organism survives longer in some of the infected hosts than is assumed to be the case in yellow fever in man.

#### SUMMARY.

Examinations of fresh blood from yellow fever patients by means of the dark-field microscope, made in more than twenty-seven cases,



revealed in three cases the presence of *Leptospira icteroides*. In no instance was a large number of organisms found, a long search being required before one was encountered. The injection of the blood into guinea pigs from two of the three positive cases induced in the animals a fatal infection, while the blood from the third positive case failed to infect the guinea pigs fatally. Careful but by no means exhaustive dark-field searches for the leptospira with fresh specimens of blood from the remaining cases of yellow fever ended without positive findings, although four of the specimens, when injected into guinea pigs, caused a fatal leptospira infection.

Stained blood film preparations from the corresponding cases were also examined, but the percentage showing the leptospira in the blood was no greater than that found by examination in the fresh state with the dark-field microscope. In fact, owing to the defective stains that were available at the time of the investigation a great many slides did not take the proper coloration with Giemsa's or Wright's stain and could not be relied upon.

Regarding the presence of *Leptospira icteroides* in various organs both dark-field and stained films were examined. In only one instance so far a few organisms were detected in the emulsion of liver taken shortly after death from a case dying on the 4th day of yellow fever. This part of the work will be reported later upon completion.

Examinations of the urine from different cases of yellow fever were made both by dark-field microscope and by inoculation into guinea pigs. The results were totally negative in thirteen cases, including many convalescents, but in one case one of the guinea pigs inoculated with 10 cc. of the urine came down on the 15th day with suggestive symptoms (suspicion of jaundice, and some hemorrhagic and parenchymatous lesions of the lungs and kidneys). This specimen showed no leptospira by dark-field examination.

In experimental infection of guinea pigs with *Leptospira icteroides* the blood became infective in many instances 48 hours after inoculation, and was always infective after 72 hours. The liver and kidney become infective simultaneously with the blood. Detection of the organism by means of the dark-field microscope has seldom been accomplished before the 5th day. The organisms are most abundant on the 6th to the 7th day, but become fewer or completely

disappear before death. In the meanwhile the number of organisms increases in the liver and kidney, from which they disappear as the jaundice and other symptoms become aggravated. When death occurs these organs seem to have lost most of the leptospira, and positive transfer by means of them is less certain. At the later stage of the disease the blood is often free from the organisms and ceases to be infective. Positive transmission with blood obtained from moribund animals is not impossible, however, even when no leptospira can be detected under the dark-field microscope.



## ETIOLOGY OF YELLOW FEVER.

### VIII. PRESENCE OF A LEPTOSPIRA IN WILD ANIMALS IN GUAYAQUIL AND ITS RELATION TO LEPTOSPIRA ICTEROHÆMORRHAGIÆ AND LEPTOSPIRA ICTEROIDES.

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*Leptospira icterohæmorrhagiæ* was demonstrated in the kidneys of wild rats first by Ido and his associates in Japan,<sup>1</sup> then by Stokes, Ryle, and Tytler,<sup>2</sup> Courmont and Durand,<sup>3</sup> Martin and Pettit,<sup>4</sup> Renaux,<sup>5</sup> Coles,<sup>6</sup> Monti,<sup>7</sup> Grasso,<sup>8</sup> and Dalmau<sup>9</sup> in Europe; and by Noguchi<sup>10</sup> and Jobling and Eggstein<sup>11</sup> in North America; by Nicolle and Lebaillly<sup>12</sup> in Tunis, and by Lhéritier<sup>13</sup> in Algeria. That the leptospira found in wild rats is in all probability identical with that which produces infectious jaundice in man was the conclusion of Ido and his associates<sup>1</sup> and later of the writer<sup>10</sup> from the reciprocal immunity reactions between the human and rat strains. Just how the rat strain enters the human body is still a

<sup>1</sup> Ido, Y., Hoki, R., Ito, H., and Wani, H., *J. Exp. Med.*, 1917, xxvi, 341.

<sup>2</sup> Stokes, A., Ryle, J. A., and Tytler, W. H., *Lancet*, 1917, i, 142.

<sup>3</sup> Courmont, J., and Durand, P., *Bull. et mém. Soc. méd. hôp. Paris*, 1917, xli, 115.

<sup>4</sup> Martin, L., and Pettit, A., *Compt. rend. Soc. biol.*, 1917, lxxx, 10, 574; 1918, lxxxi, 697.

<sup>5</sup> Renaux, E., *Compt. rend. Soc. biol.*, 1917, lxxx, 405.

<sup>6</sup> Coles, A. C., *Lancet*, 1918, i, 468.

<sup>7</sup> Monti, A., *Boll. Soc. med.-chir. Pavia*, 1917, cited by Martin, L., and Pettit, A., *Spirochétose ictérohémmorrhagique*, Monographies de l'Institute Pasteur, Paris, 1919.

<sup>8</sup> Grasso, G., *Pathologica*, 1918, x, 8.

<sup>9</sup> Dalmau, M., *Treballs Soc. Barcelone*, 1918, cited by Martin, L., and Pettit, A., *Spirochétose ictérohémmorrhagique*, Monographies de l'Institute Pasteur, Paris, 1919.

<sup>10</sup> Noguchi, H., *J. Exp. Med.*, 1917, xxv, 755.

<sup>11</sup> Jobling, J. W., and Eggstein, A. A., *J. Am. Med. Assn.*, 1917, lxix, 1787.

<sup>12</sup> Nicolle, C., and Lebaillly, C., *Compt. rend. Soc. biol.*, 1918, lxxxi, 349.

<sup>13</sup> Lhéritier, A., *Bull. Soc. path. exot.*, 1918, xi, 357.



matter of conjecture, based on circumstantial evidence. It is not yet entirely clear how the leptospira happens to be carried by rats. It is probable that the infection is an accidental one, occasioned by the direct transmission of the leptospira to man through exposure of some part of the body to the water of a cess-pool or to moistened ground on which the urine of an infected rat has been deposited within a few hours previously. In a longer period the organisms would be destroyed by other saprophytic bacteria.<sup>14</sup> The incidence of infection is much greater in countries where the body is exposed, especially the feet and hands, to ground or water infested by wild rats. The occupation of the people also plays an important part in the frequency of infection. Once a rat becomes infected it supplies the infective agent for an indefinite length of time, possibly until its death. Transmission of the leptospira from rat to rat is accomplished by infected food or drink and is particularly easy in view of their cannibalism.

The question of the presence of a leptospira in tropical countries, especially where endemic foci of yellow fever exist, has not heretofore been studied, but with the isolation of a pathogenic leptospira from certain cases of yellow fever in Guayaquil the relation between the human and animal strains demands a thorough investigation. A general survey has therefore been made to detect the presence of the leptospira among the wild animals encountered in Guayaquil.

The mode of study consisted in inoculating intraperitoneally 1 to 2 cc. of a Ringer solution emulsion of both kidneys of two animals into three guinea pigs. The emulsions were prepared, in the proportion of 1 gm. of the kidney to 10 cc. of Ringer solution, by finely grinding up the kidneys in a mortar with sterile sand. Rats, mice, bats, and an opossum were examined for the presence of the leptospira. These animals are abundant in and about the houses and buildings in Guayaquil, and they were caught and sent in alive by the Board of Health<sup>15</sup> of the city. Table I shows the results of the investigation.

The experiments show that eight out of twelve groups of rats carried in their kidneys a leptospira capable of producing in guinea pigs pathological changes similar to those produced by *Leptospira icterohæmorrhagiæ*. Only two groups of rats were absolutely free

<sup>14</sup> Noguchi, H., *J. Exp. Med.*, 1918, xxvii, 593.

<sup>15</sup> I am greatly indebted to Dr. León Becerra of the Board of Health, for his cooperation in the course of the work which was carried on in Guayaquil. For some specimens of bats I am also indebted to Dr. Gonzalez-Rubio, Jr.

TABLE I.  
*Experiments with Rats.*

Groups of rats.	Guinea pig No.	Results.	Remarks.
1 (doubtful).	373	Temperature 40.2°C. on 5th day. Survived.	Perhaps an abortive infection.
	374	Temperature 39.8°C. on 5th day. Survived.	“ “
	375	Apparently no reaction.	Negative.
2 (positive).	376	Temperature 40.4°C. on 6th day. Epistaxis and icterus on 7th day. Killed. Lungs and stomach hemorrhagic. Liver icteric and enlarged. Acute parenchymatous nephritis. Other organs unchanged. No leptospira found by dark-field examination.	Transfer to Guinea Pig 377 A (blood 2 cc.). Temperature 40°C. on 5th day. Killed “ 8th “ Typical, but no leptospira found by dark-field examination.
	376 A	Apparently no febrile reaction. Animal became icteric with temperature of 36.2°C. on 8th day. Killed. Findings typical; difficult to find leptospira in blood or organs.	In a later passage the leptospira was demonstrated.
	377	Died over night.	
3 “	378	Only slight temperature rise, but typically jaundiced on 8th day and was killed. Typical changes of the organs. Leptospira difficult to find.	
	338 A	Died in 9 days without typical changes.	
	389	Died over night.	
4 “	380	Became jaundiced on 5th day. Died in 9 days. Typical findings. Leptospira not demonstrated.	
	381	Temperature 40.2° on 10th day; 40°C. on 11th day, but animal never came down with typical symptoms.	
	382	Temperature 40.5°C. on 8th day and 37.4° on 10th day, with extreme jaundice. Typical findings with leptospira in the tissues.	

TABLE I—*Continued.*

Groups of rats.	Guinea pig No.	Results.	Remarks.
5 (negative).	383	Died of intercurrent infection in 10 days.	
	384	No reaction.	
	385	Temporary rise in temperature for several days. Survived.	
6 “	386	Died in 2 days. Negative.	
	386 A	Temperature 40.3°C. on 7th day, but with ultimate recovery.	
	387	No reaction.	
7 (doubtful).	388	Nothing typical.	Perhaps an abortive infection.
	388 A	Temperature 40.2°C. on 4th day, but recovered.	
	389	Died in 14 hours. Negative.	
8 (positive).	390	Temperature 40.2° on 5th day, 38.6°C. on 7th, with marked jaundice. Killed. Typical findings in the organs; leptospira not demonstrated by dark-field examination.	Transfer to two guinea pigs (blood 2 cc.). Both became typically yellow and were killed on 10th day. Typical findings, with leptospira in liver, but none in kidney or blood of one animal; second animal showed no leptospira.
	390 A	Highest temperature 39°C. on 5th day. Died in 9 days. Typical lesions.	
	391	Slight febrile reaction. Recovered.	
	392	Highest temperature 39.4°C. on 5th day. Died on 7th day with typical lesions.	
	392 A	Highest temperature 39.6°C. on 8th day. Died on 10th day with icterus and hemorrhages.	
9 “	393	Temperature 40.1°C. on 6th day, 39.9° on 7th day. Transient icterus on 10th day. Recovered.	

TABLE I—*Concluded.*

Groups of rats.	Guinea pig No.	Results.	Remarks.
10 (positive).	394	No high fever. Icterus in 8 days. Killed in 11 days. Lesions typical. <i>Leptospira</i> difficult to find.	Transfer to two guinea pigs. Both died in 7 days with typical lesions and jaundice.
	394 A	Temperature 39.4°C. on 5th day. Icterus following day. Killed on 8th day. Typical lesions at autopsy. <i>Leptospira</i> found.	
	395	Remained well.	
11 “	396	Temperature 39.7°C. on 4th, 40° on 5th, 36.6° on 7th day. Intensely jaundiced. Killed in 6 days. Typical changes. <i>Leptospira</i> difficult to find.	Transfer to two guinea pigs (blood 2 cc. each). One died in 7 and the other in 9 days—both intensely jaundiced. Typical lesions. Melena and black vomit in latter.
	396 A	Had very little fever, but died with jaundice in 9 days. Autopsy typical.	
	397	Temperature 39.6–39.8°C. on 6th, 7th, and 8th days. Suspicion of icterus. Killed for examination in 15 days. Lungs showed old hemorrhagic areas; other organs not changed.	This animal was convalescing after a mild infection.
12 “	398	Remained well.	
	399	“ “	
	400	Died in 13 days, with typical jaundice and lesions.	

from the organism, while two others showed a suspicious reaction without terminating in fatal infection. In other words, about 67 per cent of the rats studied harbored the *leptospira* in their kidneys.

In testing mice for the same organism it was found that out of three groups of seven mice each, one produced extreme jaundice and the other typical changes in two out of three guinea pigs, one dying in 8 and the other in 9 days after the inoculation of the kidney emulsion. There was hemorrhage before death from the rectum in one



and from the nose in the other. The leptospira was difficult to find in the blood, liver, and kidney, but was found in subsequent passages.

No positive results were obtained with the kidney emulsions of eight bats and one opossum. It may be noted that Nicolle and Lebailly<sup>12</sup> obtained negative results with bats caught in Tunis.

The experiments show that the emulsions which produced a fatal infection in guinea pigs did not always kill all the guinea pigs inoculated with the same quantities of the same material and under the same conditions. Some died, while others had a mild abortive infection or escaped infection altogether. This seems to indicate that there exists among individual guinea pigs a considerable variation in their susceptibility to the same strain of the organism and explains why it is important to use as many guinea pigs as convenient for the purpose of transmitting the organism to this animal. There is a close analogy with the frequent abortive infections which were obtained in the attempts to transmit *Leptospira icteroides* to guinea pigs.

The relation between the rodent strains and the strains of *Leptospira icteroides* on the one hand and those of *Leptospira icterohæmorrhagiæ* on the other was next studied from the standpoint of pathogenicity and immunity. For this purpose two strains of leptospira isolated from rats and one from mice were used. The three strains, designated Groups 8, 11, and 30, have morphological features identical with those of the strains isolated from wild rats caught in the vicinity of New York,<sup>10</sup> and are consequently indistinguishable from the icterohemorrhagic strains derived from the Japanese and European sources. They are slightly coarser than the strains of *Leptospira icteroides*.

With regard to the pathogenicity of the Guayaquil rat leptospira, it is difficult to point out any essential difference between the symptoms and lesions that occur in guinea pigs infected with it and those in animals inoculated with the icterohemorrhagic strains of temperate climates. They all produce jaundice and hemorrhages, although with some strains hemorrhage is the outstanding feature.

*Identification of the Organism by Means of Immunity Reactions.*

In order to determine whether the leptospira isolated from wild rodents in Guayaquil is identical with the strains of *Leptospira icterohæmorrhagiæ* from other sources, and what relation it may have to *Leptospira icteroides* from yellow fever patients in the same city, their immunity reactions were taken into consideration in a series of experiments.

*Immune Sera.*—Two rabbits were immunized with each of the three strains of Guayaquil rodent leptospira by injecting intravenously 2 to 4 cc. of rich living cultures of the organisms several times at 7 to 12 day intervals. The animals were bled on the 9th day after the last injection and the effects of their sera tested not only upon the same and other strains of Guayaquil origin, but also upon the Japanese, European, and New York strains of *Leptospira icterohæmorrhagiæ*. The relation of this group of organisms to that of *Leptospira icteroides* was likewise studied and will be discussed at greater length in connection with the relation between yellow fever and infectious jaundice.

The first experiments were designed for observation of the action of each of these sera upon the organism *in vitro*. To 0.5 cc. of a rich suspension of culture in saline solution was added 0.2 cc. of the immune serum with or without the simultaneous addition of 0.2 cc. of fresh normal guinea pig serum as complement. The mixture was placed in a water bath at 37°C. for 2 hours and then examined under the dark-field microscope. The entire procedure was carried out with strict aseptic precautions, and each experiment was accompanied by a control with normal rabbit serum. The tubes were kept at room temperature for 96 hours and their contents examined again. Except for a greater amount of precipitation in some tubes the results were about the same as those observed at the end of 2 hours at 37°C. The control tubes with normal rabbit serum showed numerous active organisms after 4 days.

Pfeiffer's phenomenon was also studied by the usual procedure; that is, examination of the peritoneal fluid of guinea pigs after inoculation with a given serum and the strain in question. In this instance 0.5 cc. of rich culture was mixed with 1 cc. of the immune serum and

injected intraperitoneally, examination being made after 30 minutes and 2 hours.

As another means of identification the protective property of each immune serum (1 cc.) was tested on guinea pigs against approximately 10 minimum lethal doses of the different strains. Unfortunately

TABLE II.

*Immunological Relation of the Guayaquil Rat Leptospira to Leptospira icterohæmorrhagiæ.*

Immune Serum 906, produced with Group 8 strain, injected on Jan. 15, 22, Feb. 3, 15, 1919.

Cultures tested.	Effects <i>in vitro</i> .	Pfeiffer phenomenon.	Protective property against infection in guinea pigs.
Group 8	Marked precipitation, agglutination, and disintegration.	Positive.	1 cc. protected guinea pigs against about 10 minimum lethal doses.
" 11	Effects similar to the foregoing but less in degree.	"	" "
" 30	Marked agglutination and later disintegration.	"	" "
Japanese.	Similar to the foregoing but less marked.	"	" "
American No. 1	Rather marked agglutination.	"	Could not be tested because of loss of virulence.
" " 3	Effects similar to the foregoing.	"	" "
French.	" "	"	1 cc. protected guinea pigs against about 10 minimum lethal doses.

the virulence of the American and British strains of *Leptospira icterohæmorrhagiæ* was considerably attenuated during my absence of 6 months and could not be tested with reliable results. This, however, was not a serious obstacle to determining the affinity of these strains for the Guayaquil strains, because an immune serum produced in rabbits with the avirulent American strain was tested against the pathogenic Guayaquil strains. A brief summary of the foregoing experiments is given in Tables II to VI.

TABLE III.

*Immunological Relation of the Guayaquil Rat Leptospira to Leptospira icterohæmorrhagiæ.*

Immune Serum 914, produced with Group 11 strain, injected on Dec. 30, 1918, Jan. 6, 14, 21, 1919.

Cultures tested.	Effects <i>in vitro</i> .	Pfeiffer phenomenon.	Protective property against infection in guinea pigs.
Group 8	Moderate agglutination but no degeneration.	Positive.	1 cc. protected guinea pigs against about 10 minimum lethal doses.
" 11	Marked precipitation and agglutination; later degeneration.	"	" "
" 30	Effects similar to the foregoing but less marked.	"	" "
Japanese.	" "	"	" "
American No. 1	" "	"	Could not be tested because of loss of virulence.
" " 3	Marked agglutination and degeneration.	"	" "
French.	" "	"	1 cc. protected guinea pigs against about 10 minimum lethal doses.

TABLE IV.

*Immunological Relation of the Guayaquil Rat Leptospira to Leptospira icterohæmorrhagiæ.*

Immune Serum 904, produced with Group 30 strain, injected on Dec. 30, 1918, Jan. 6, 14, 22, Feb. 3, 15, 1919.

Cultures tested.	Effects <i>in vitro</i> .	Pfeiffer phenomenon.	Protective property against infection in guinea pigs.
Group 8	Marked agglutination and subsequent disintegration.	Positive.	1 cc. protected guinea pigs against about 10 minimum lethal doses.
" 11	Slight agglutination, but no degeneration.	"	" "
" 30	Marked precipitation, agglutination, lysis, and degeneration.	"	" "
Japanese.	" "	"	" "
American No. 1	" "	"	Could not be tested on account of loss of virulence.
" " 3	Slight agglutination; no degeneration.	"	" "
British.	" "	"	" "



TABLE V.

*Immunological Relation of the Guayaquil Rat Leptospira to Leptospira icterohæmorrhagiæ.*

Immune Serum 952, produced with American Strain 1, injected on Dec. 30, 1918, Jan. 6, 14, 22, Feb. 3, 15, 1919.

Cultures tested.	Effects <i>in vitro</i> .	Pfeiffer phenomenon.	Protective property against infection in guinea pigs.
Group 8	Moderate agglutination; slight degeneration.	Positive.	1 cc. protected guinea pigs against about 10 minimum lethal doses.
" 11	Very marked agglutination and degeneration.	"	Not tested.
" 30	Slight agglutination and degeneration.	"	1 cc. protected guinea pigs against about 10 minimum lethal doses.
Japanese.	Moderate agglutination and degeneration.	"	Not tested.
American No. 1	Marked agglutination and degeneration.	"	" "
British.	Slight agglutination and degeneration.	"	" "

TABLE VI.

*Immunological Relation of the Guayaquil Rat Leptospira to Leptospira icterohæmorrhagiæ.*

Immune Serum 911, produced with the Japanese strain, injected on Dec. 30, 1918, Jan. 6, 12, 22, Feb. 3, 15, 1919.

Cultures tested.	Effects <i>in vitro</i> .	Pfeiffer phenomenon.	Protective property against infection in guinea pigs.
Group 8	Moderate agglutination and degeneration.	Positive.	1 cc. protected guinea pigs against about 10 minimum lethal doses.
" 11	Slight agglutination; no degeneration.	"	" "
" 30	Marked agglutination and disintegration.	"	" "
Japanese.	Very marked agglutination and lysis.	"	" "
American No. 1	Moderate agglutination and degeneration.	"	Could not be tested because of loss of virulence.
British.	Slight agglutination and degeneration.	"	" "

The tables show that while there exist undeniable variations in the intensity of the immunity reactions as manifested *in vitro* in the form of agglutination and subsequent disintegration of the organism, as well as *in vivo* in Pfeiffer's phenomenon and protection against infection with one or the other strains, the variations are nevertheless of so slight a nature as to lead one to assume that the strains isolated

TABLE VII.

*Immunological Relation of the Guayaquil Rat Leptospira to Leptospira icteroides.*

Immune Serum 906, produced with Group 8 strain, injected on Jan. 15, 22, Feb. 3, 15, 1919.

<i>Leptospira icteroides</i> strain.	Effects <i>in vitro</i> .	Pfeiffer phenomenon.	Protective property against infection in guinea pigs.
A. A.	Slight agglutination; no degeneration.	Negative.	No protection.
E. Ch.	No effect.	"	" "
A. Ce.	" "	"	" "

TABLE VIII.

*Immunological Relation of the Guayaquil Rat Leptospira to Leptospira icteroides.*

Immune Serum 914, prepared with Group 11 strain, injected on Dec. 30, 1918, Jan. 6, 14, 21, 1919.

<i>Leptospira icteroides</i> strain.	Effects <i>in vitro</i> .	Pfeiffer phenomenon.	Protective property against infection in guinea pigs.
A. A.	No effect.	Negative.	No protection.
E. Ch.	" "	"	" "
A. Ce.	" "	"	" "
M. G.	Definite agglutination, but no immobilization.	Doubtful.	1 cc. did not prevent the infection, but the animal survived.

from rats and mice in Guayaquil belong to the group of *Leptospira icterohæmorrhagiæ* now known to be widely distributed among these rodents inhabiting the temperate zone.

The results of a parallel series of experiments performed with the immune sera on different strains of *Leptospira icteroides* are given in Tables VII to IX.

*Prophylactic Inoculation.*—February 6, 1919. A number of guinea pigs were inoculated subcutaneously with 0.5 and 2 cc. of killed culture (heated to 60°C. for 10 minutes in the water bath) of the Guayaquil rat strains of leptospira, Groups 8, 11, and 30. These animals were inoculated after 15 days (February 21) with virulent cultures of the same and of other strains. At the same time some of the vaccinated guinea pigs were inoculated also with the icterohemorrhagic strains, including the American No. 1,<sup>16</sup> the Japanese, and the

TABLE IX.

*Immunological Relation of the Guayaquil Mouse Leptospira to Leptospira icteroides.*

Immune Serum 904, produced with Group 30 strain, injected on Dec. 20, 1918, Jan. 6, 14, 22, Feb. 3, 15, 1919.

<i>Leptospira icteroides</i> strain.	Effects <i>in vitro</i> .	Pfeiffer phenomenon.	Protective property against infection in guinea pigs.
E. Ch.	Slight agglutination without immobilization.	Doubtful.	No protection.
A. Ce.	No effect.	Negative.	" "
M. G.	" "	"	" "
A. A.	" "	"	" "

French. The guinea pigs previously inoculated with the killed cultures of the Guayaquil strains proved resistant to a subsequent infection, not only with homologous but also with the heterologous strains derived from Japanese, European, and American sources. This experiment indicates that the Guayaquil rodent strains of leptospira are identical with *Leptospira icterohæmorrhagiæ*.

## SUMMARY.

By the inoculation of guinea pigs intraperitoneally with the emulsions of kidneys from wild rats and mice captured in Guayaquil, it was found that 67 per cent of the wild rats tested harbored in their kidneys a leptospira which produced in guinea pigs symptoms and lesions identical with those produced by *Leptospira icterohæmorrhagiæ* derived either from patients suffering from infectious jaundice in Japan or Europe, or from wild rats caught in New York.

<sup>16</sup> This strain was so attenuated that some of the guinea pigs escaped a fatal infection and could not be used in further experiments.

Immune sera were prepared in rabbits by injecting different strains of the Guayaquil leptospira. These sera had a marked agglutinating and disintegrating influence upon the homologous strains, and also, but often to a less pronounced degree, upon the strains of *Leptospira icterohæmorrhagiæ* from other sources. Pfeiffer's phenomenon was also found to be positive, and protection was demonstrated against infection with virulent cultures of strains of *Leptospira icterohæmorrhagiæ*.

The same sera had no effect, or at most a very slight one, upon *Leptospira icteroides*. Guinea pigs inoculated with *icteroides* strains were not noticeably protected by the use of the immune sera prepared with the Guayaquil rat strains.

Guinea pigs inoculated with killed cultures of the Guayaquil strains of leptospira proved to be resistant to a subsequent infection with heterologous as well as homologous strains of *Leptospira icterohæmorrhagiæ*.

It is concluded, therefore, that the leptospira isolated from the kidneys of wild rats and mice in Guayaquil belongs to the group of *Leptospira icterohæmorrhagiæ*, and differs from *Leptospira icteroides* in its immunity reactions.

No positive transmission was obtained with kidney material from bats and an opossum.





## THE PASSAGE OF MENINGOCOCCIC AGGLUTININS FROM THE BLOOD TO THE SPINAL FLUID OF THE MONKEY.

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The administration of antimeningococcic serum for the treatment of epidemic meningitis by other than the intraspinal route has hardly been considered until recently, since the publication of the papers by Flexner and his coworkers<sup>1,2</sup> on the specific serum therapy of the disease on which the prevailing mode of treatment is based. The experience with a large number of cases of epidemic meningitis during the great war and under the unusual conditions of camp life has led to the revival of the employment of the serum by intravenous, usually associated with intraspinal, injection. That the intravenous administration was both indicated and called for is evidenced by the occurrence of cases of meningococcemia in some instances without, and in others before the onset of, the meningitis.<sup>3,4</sup> Although other clinicians have from time to time employed antimeningococcic serum by intravenous injection the systematic use of it in that manner has been developed especially by Herrick.

The purpose of the intravenous injection may be regarded as threefold: (1) to combat the meningococcemia; (2) to diminish possibly the rapidity of the passage of the serum from the subarachnoid space into the blood; and (3) to bring remote portions of the meninges, not so readily accessible from the spinal fluid itself, under the influ-

<sup>1</sup> Flexner, S., *J. Am. Med. Assn.*, 1906, xlvii, 560; *J. Exp. Med.*, 1917, ix, 168; 913, xvii, 553.

<sup>2</sup> Flexner, S., and Jobling, J. W., *J. Exp. Med.*, 1908, x, 141, 690.

<sup>3</sup> Herrick, W. W., *J. Am. Med. Assn.*, 1918, lxxi, 62; *Arch. Int. Med.*, 1918, xxi, 541.

<sup>4</sup> Baeslack, F. W., Bunce, A. H., Brunelle, G. C., Fleming, J. S., Klugh, G. F., McLean, E. H., and Salomon, A. V., *J. Am. Med. Assn.*, 1918, lxx, 684.

ence of the serum. Under normal conditions antibodies do not pass from the blood into the cerebrospinal fluid,<sup>5</sup> but under circumstances of inflammation of the meninges the permeability is increased, and passage may in some degree take place. Of the three possibilities, the first, namely the control of the blood invasion by meningococci, may be regarded as the most important, since it may, if only in rare instances, prevent or abort a meningeal infection, and it may be even more effective in preventing the infections of joints, heart, eye, etc.

The experiments to be described relate to the question of the possibility and the degree of passage of antibodies, in this instance agglutinins, for the meningococcus from the blood into the cerebrospinal fluid.

#### EXPERIMENTAL.

*Macacus rhesus* monkeys were employed for the experiments because of the ease with which chemical meningitis may be induced in them and especially because they readily yield cerebrospinal fluid on lumbar puncture.

The first experiment was arranged to test two points: (a) whether the antimeningococcus agglutinins passed from the blood into the cerebrospinal fluid in normal animals, and (b) whether they passed in animals in which a chemical meningitis had been incited when only 10 cc. of the antiserum had been injected into a superficial vein. The protocol which follows shows not only that no such passage takes place in the normal animal but either none that is demonstrable, or at least very little, even in the presence of an aseptic meningitis.

*Experiment 1.*—Mar. 13, 1918. Three *Macacus rhesus* monkeys, weighing about 3 kilos each, received intravenously 10 cc. of polyvalent antimeningococcic serum. Monkeys A and B had received 18 hours previously 2 cc. of normal horse serum intraspinally. No intraspinal injection was given Monkey C. 7 and 24 hours after the intravenous injection spinal fluid was removed from each and tested for agglutination against the meningococcus. None was observed in either specimen from the monkey which received no intraspinal injection. The same was true of the specimens from one of the monkeys in which chemical meningitis had been induced. The spinal fluid removed 7 hours but not 24 hours after intravenous injection from the second monkey, which had received intraspinal injection of horse serum, agglutinated regular and parameningococcus in a dilution of 1:2 and 1:4.

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<sup>5</sup> Flexner, S., *J. Am. Med. Assn.*, 1913, lxi, 447, 1872.

The second experiment was made with 20 cc. of polyvalent anti-meningococcic serum of high titer which was injected in each instance into a superficial vein. It was devised to cover the following conditions: (a) effect on normal cerebrospinal fluid, (b) effect on cerebrospinal fluid modified by a mild aseptic meningitis induced by an intraspinal injection of sterile normal salt solution, (c) effect on the cerebrospinal fluid modified by a more severe chemical inflammation induced by normal horse serum, and (d) a still more severe inflammation brought about by the injection of a salt solution suspension of regular meningococcus culture. In the last instance the agglutinin tested for was that of the para organism.

The result is clear: no agglutinins were present in the cerebrospinal fluid derived from the normal animal which received the intravenous injection; fluctuating amounts were present in the cerebrospinal fluid after salt solution, more after the horse serum injection, and most after the injection of meningococcus. In other words, the normal meninges were not permeable, while the inflamed membranes were permeable in proportion to the intensity of the meningitis experimentally induced.

*Experiment 2.*—*Macacus rhesus* D, weight 3.2 kilos; control. Mar. 21, 1918. Injected intravenously 20 cc. of polyvalent antimeningococcic serum. 5.25 p.m. Lumbar puncture, 0.5 cc. of clear fluid which did not agglutinate regular or parameningococcus. Mar. 22, 10.10 a.m. Lumbar puncture, 1 cc. of clear fluid which did not agglutinate the meningococci. 5.50 p.m. Injected intraspinally one-quarter of an 8 hour culture of regular meningococcus in 0.5 cc. of isotonic salt solution. Mar. 23, 12 m. Lumbar puncture, 0.5 cc. of turbid fluid which, after centrifugation, agglutinated parameningococcus ++ in a dilution of 1:4. Culture from spinal fluid negative. Mar. 24, 10.35 a.m. Lumbar puncture, 0.5 cc. of turbid fluid. Cultures negative. Centrifuged fluid agglutinated parameningococcus + in a dilution of 1:2.

*Macacus rhesus* E, weight 3.5 kilos. Mar. 20, 1918, 4 p.m. Injected intraspinally 2 cc. of isotonic salt solution. Mar. 21, 11.10 a.m. Injected intravenously 20 cc. of polyvalent antimeningococcic serum. Mar. 22, 10.30 a.m. Lumbar puncture, 1 cc. of slightly turbid fluid. The centrifuged fluid did not agglutinate regular or parameningococcus in a dilution of 1:2. 5.45 p.m. Injected intraspinally one-quarter of an 8 hour culture of regular meningococcus in 0.5 cc. of isotonic salt solution. Mar. 23, 11 a.m. Lumbar puncture, 0.5 cc. of turbid fluid which yielded negative culture. The centrifuged fluid agglutinated parameningococcus + in a dilution of 1:4. Mar. 24, 10.25 a.m. Lumbar punc-



ture, 0.5 cc. of slightly turbid fluid which yielded negative culture. The centrifuged fluid agglutinated parameningococcus ++ in a dilution of 1:4.

*Macacus rhesus* F, weight 3.3 kilos. Mar. 20, 1918, 4 p.m. Injected intraspinally 2 cc. of normal horse serum. Mar. 21, 10.38 a.m. Injected intravenously 20 cc. of polyvalent antimeningococcic serum. Mar. 22, 10.20 a.m. Lumbar puncture, 0.5 cc. of turbid spinal fluid. The centrifuged fluid agglutinated regular meningococcus = 1:4, - 1:2, and parameningococcus + 1:4 and - 1:2. 6.15 p.m. Lumbar puncture, 0.5 cc. of turbid fluid which after centrifuging agglutinated parameningococcus + 1:4. Injected intraspinally one-fourth of an 8 hour culture of regular meningococcus in 0.5 cc. of isotonic salt solution. Mar. 23, 11.30 a.m. Lumbar puncture, 0.5 cc. of turbid fluid; culture negative; the centrifuged fluid agglutinated regular meningococcus + in a dilution of 1:2, ++ 1:4, and parameningococcus + 1:2, - 1:4.

The next experiment brings out conclusively the effect of degree of chemical inflammation in promoting the passage of agglutinating bodies from the blood into the spinal fluid. The order of the experiment was first to inject intraspinally into *Macacus rhesus* monkeys either isotonic salt solution or normal horse serum, and about 20 hours later to give 20 cc. of polyvalent antimeningococcic serum intravenously. The spinal fluid is then withdrawn at stated intervals and tested. When salt solution or horse serum is injected intraspinally and the turbid fluid withdrawn later and tested for agglutinins of the meningococcus no agglutination takes place unless antimeningococcic serum has been given also. The results of this experiment are given in Table I. The far greater effect of the horse serum over isotonic salt solution is at once apparent.

Attention has been drawn to the practice now becoming more common of combining the intravenous with the intraspinal injection of the antimeningococcic serum. Time and experience alone will decide in how far this practice is superior to the intraspinal injection alone. The serum introduced into the subarachnoid space soon begins to escape into the blood; hence the necessity for its reintroduction if the meninges are to be kept bathed in the antiserum. The passage of the antiserum from the subarachnoid space into the blood leads to a general distribution throughout the organs. Possibly the higher dilution thus produced compared with the greater concentration secured from a direct injection of the larger volume of serum into the blood gives to the latter a special advantage in overcoming the so called meningococcemia.

It seemed desirable to determine, by direct agglutination tests, the agglutination titer of the blood and spinal fluid under the three sets of conditions; namely, after an intraspinal injection alone, after an intravenous injection alone, and after combined intraspinal and intravenous injection. This determination was made in monkeys; it could of course be carried out better with cases of epidemic meningitis under serum treatment. Unfortunately we were restricted by the scarcity of monkeys to one test of each condition. The result given in Text-fig. 1, *a*, *b*, and *c* must be regarded, therefore, as tentative only. Certain factors affecting the results are entirely ob-

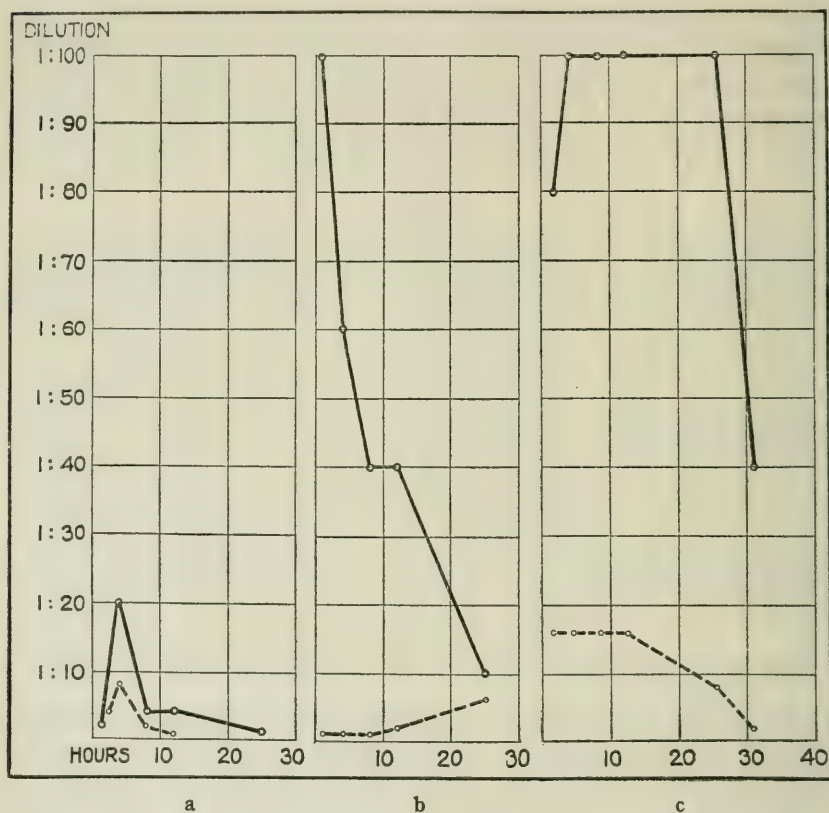
TABLE I.

Intraspinal injection.	Length of time after intravenous injection.	Dilutions of spinal fluid.					
		1:2	1:4	1:8	1:10	1:20	1:40
Isotonic salt solution.	<i>hrs.</i>						
	20	+	+	±	—	—	—
	25	+	+	±	—	—	—
	45	±	±	—	—	—	—
	69	±	—	—	—	—	—
	93	±	—	—	—	—	—
Normal horse serum.	20	+	+	+	±	—	—
	25	+	+	+	+	±	—
	45	++	++	++	+	+	+
	69	±	+	±	—	—	—
	93	+	+	±	—	—	—

scure; such, for example, as the delay after intravenous injection before the full expression of the agglutination titer declares itself. The three curves are, however, not only distinct, but suggest first that passage of the serum from the cerebrospinal fluid into the blood begins almost at once (Text-fig. 1, *a*), and second that the persistence of the titer in the blood and spinal fluid (Text-fig. 1, *c*) is sensibly affected by combined intraspinal and intravenous injection. However, we desire to repeat that not too much stress should be laid upon the results of these single tests.

*Experiment 3.*—*Macacus rhesus* G. May 14, 1918, 9.15 a.m. Injected intraspinally 3 cc. of polyvalent antimeningococcic serum. Blood was taken for

agglutination tests at 10.30 a.m., 1.15, 5.15, and 9.30 p.m. Lumbar puncture at 10.50 a.m., 1.25, 5.45, and 9.45 p.m. May 15. Bleedings at 10.45 a.m. and 4.18 p.m. Lumbar puncture at 10.50 a.m. and 4.23 p.m. The samples were tested May 16 for agglutinins with a parameningococcus, and the results are recorded in Text-fig. 1, *a*.



— Meningococcic agglutinins in the blood.  
 - - - - - Meningococcic agglutinins in the spinal fluid.

TEXT-FIG. 1, *a*, *b*, and *c*. (*a*) Meningococcic agglutinins in the blood and spinal fluid of the monkey after intraspinal injection of 3 cc. of antimeningococcic serum. (*b*) Meningococcic agglutinins in the blood and spinal fluid of the monkey after intravenous injection of antimeningococcic serum and the intraspinal injection of normal horse serum. (*c*) Meningococcic agglutinins in the blood and spinal fluid of the monkey after combined intravenous and intraspinal injection of antimeningococcic serum.

*Macacus rhesus* H. May 13, 1918, 5 p.m. Injected intraspinally 2 cc. of normal horse serum. May 14, 8.10 a.m. Injected intravenously 20 cc. of polyvalent antimeningococcic serum. Specimens of spinal fluid and blood were taken at 9.10 a.m., 12.10 and 8.10 p.m., and at 9.10 a.m. on May 15. The results of the agglutination tests with parameningococcus on May 16 are recorded in Text-fig. 1, *b*.

*Macacus rhesus* I. May 14, 1918, 9.30 a.m. Injected intraspinally 3 cc. and intravenously 20 cc. of polyvalent antimeningococcic serum. Samples of blood and spinal fluid were removed at 11 a.m., 1.30, 6, and 9.40 p.m. May 15. Specimens taken at 10.50 a.m. and 4.25 p.m. The results of agglutination tests with parameningococcus on May 16 are shown in Text-fig. 1, *c*.

#### CONCLUSIONS.

Agglutinins for the meningococcus were not found in the spinal fluid of normal monkeys which had received antimeningococcic serum intravenously.

The intraspinal injection of isotonic salt solution, normal horse serum, or a culture of living meningococci allows agglutinins for the meningococcus to pass from the blood to the spinal fluid of the passively immunized monkey; and the rate of the passage is affected by the severity of the inflammation induced in the meninges.

The rates of elimination from the blood and spinal canal of meningococcic antibodies, as shown by the agglutination reaction, were compared in monkeys treated with immune serum (*a*) intraspinally, (*b*) intravenously, and (*c*) intraspinally and intravenously in combination.

(*a*) When immune serum is given intraspinally the agglutinins are very much diminished after 8 hours and practically disappear at 12 hours. They appear in the blood at the 4th hour after injection and quickly diminish.

(*b*) After intravenous injection of immune serum, when the meninges are inflamed, agglutinins appear in the spinal fluid in small amounts in about 12 hours and increase to the 25th hour. More than one-half of the agglutinins disappear from the blood within 8 hours and remain in low concentration at 25 hours.

(*c*) After combined intraspinal and intravenous injection the agglutinins remain in higher concentration in the spinal fluid and for a longer time than by method (*a*) or (*b*). The curve descends after 12 hours, and agglutinins are present at 25 hours. They remain in maximum concentration in the blood for 25 hours.





## EXPERIMENTS ON THE MODE OF INFECTION IN EPIDEMIC MENINGITIS.

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Renewed interest has been aroused in the manner in which the meningococcus reaches the meninges from the upper respiratory mucous membrane. That the portal of entry of the meningococcus into the body is the mucosa of the nasopharynx is generally admitted. The point which has not been settled is whether the meningeal invasion is lymphatic or hematogenous. The most direct route would be along the lymphatics of the olfactory filaments to the meninges of the base of the brain; but no convincing evidence has been brought forward for that mode of invasion.

Westenhoeffer<sup>1</sup> conceived the idea that the meninges became infected from the sphenoidal sinuses; but later he abandoned that view.<sup>2</sup> Flexner<sup>3</sup> noted that when meningococci were injected intraspinally into monkeys by lumbar puncture film preparations of the nasal mucosa showed a small number of polymorphonuclear leucocytes believed to be derived from the inflamed meninges, containing Gram-negative diplococci resembling meningococci. He failed, however, to recover meningococci in cultures from the nasal mucosa of the inoculated animals, but he nevertheless discussed the possibility of a reverse process by which the meningococcus might be carried through lymphatics to the meninges in man.<sup>4</sup> This possibility was strengthened by the later observations on experimental poliomyelitis. The virus of poliomyelitis differs from the meningococcus in having a far wider range of pathogenicity for the monkey. While the meningococcus must be injected in considerable amounts directly into the subarachnoid space in monkeys in order to incite a fatal meningitis<sup>3</sup> and is entirely ineffective when

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<sup>1</sup> Westenhoeffer, *Berl. klin. Woch.*, 1905, xlii, 737.

<sup>2</sup> Westenhoeffer, M., *Berl. klin. Woch.*, 1906, xliii, 1267, 1313.

<sup>3</sup> Flexner, S., *J. Exp. Med.*, 1907, ix, 142.

<sup>4</sup> The recovery of the organism was later accomplished in these laboratories from the nasal mucosa of monkeys which had received injections of the living meningococci intraspinally.

applied to the nasal mucosa, the application of small quantities of the virus of poliomyelitis to the latter is often followed by paralysis;<sup>5</sup> on the other hand, large doses of the virus often fail to induce infection when injected directly into the blood.<sup>6</sup>

Up to the present no satisfactory solution of the problem of the mode of infection in epidemic meningitis has been secured, and no considerable additions to our knowledge of the subject have been made since the pandemic of 1905-10. The appearance of the disease among the Western Armies has again stimulated interest in the problem. Naturally enough this interest has been primarily derived from clinical observations, and efforts at a better therapeutic control of cases. The newer observations indicate that the meningococcus is more frequently present in the circulating blood than has hitherto been supposed, and, especially, that a condition of hematogenous infection with the meningococcus may precede<sup>7</sup> any demonstrable infection or inflammation of the meninges, and, finally, that it is less rare than has been believed for a general infection with the meningococcus to exist independently of meningeal involvement.<sup>8</sup> The changing therapeutic point of view brought about by

<sup>5</sup> Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1910, liv, 45.

<sup>6</sup> Clark, P. F., Fraser, F. R., and Amoss, H. L., *J. Exp. Med.*, 1914, xix, 223.

<sup>7</sup> Handa, H., and Nanjo, M., *Centr. Bakteriolog., Ref.*, 1914, lxi, 82. Bonnel and Joltrain, E., *Bull. et mêm. Soc. mêm. Hôp. Paris*, 1916, xl, 75.

<sup>8</sup> Andrewes, F. W., *Lancet*, 1906, i, 1172. Liebermeister, G., *Münch. med. Woch.*, 1908, lv, 1978. Elser, W. J., and Huntoon, F. M., *J. Med. Research*, 1909, xx, 371. Bovaird, D., *Arch. Int. Med.*, 1909, iii, 267. Monziols and Loiseleur, *Bull. et mêm. Soc. mêm. Hôp. Paris*, 1910, xxix, 155. Chevrel, F., and Bourdinière, J., *Bull. et mêm. Soc. mêm. Hôp. Paris*, 1910, xxx, 165. Cecil, R. L., and Soper, W. B., *Arch. Int. Med.*, 1911, viii, 1. Barral, Coulomb, and Couton, *Bull. et mêm. Soc. mêm. Hôp. Paris*, 1912, xxxiii, 829. Bray, H. A., *Arch. Int. Med.*, 1915, xvi, 487. Sainton, P., and Maille, J., *Bull. et mêm. Soc. mêm. Hôp. Paris*, 1915, xxxix, 296, 374. Elliott, W. M., *Lancet*, 1916, ii, 1010. Pybus, F. C., *Lancet*, 1917, i, 803. Netter, A., Salanier, M., and Wolf from, *Compt. rend. Soc. biol.*, 1916, lxxix, 973. Worster-Drought, C., and Kennedy, A. M., *Lancet*, 1917, ii, 711. Symmers, W. S., *Brit. Med. J.*, 1917, ii, 789. Anderson, W., McNee, J. W., Brown, H. R., Renshaw, A., McDonnell, J., Davidson, F. C., Gray, A. C. H., and Herringham, W. P., *J. Roy. Army Med. Corps*, 1917, xxix, 473. Sainton, P., *Paris mêm.*, 1918, viii, 86. Zeissler, J., and Riedel, F., *Deutsch. med. Woch.*, 1917, xliii, 258. Thomsen, O., and Wulff, F., *Hospitalstid.*,

these experiences has culminated in the observations just published by Herrick<sup>9</sup> and others.<sup>8</sup>

The fact, now fairly established by studies carried out very early in cases of epidemic meningitis among our recruits, that a meningococcemia may precede an actual demonstrable meningococcus invasion of the subarachnoid space, has brought up the question whether the condition favored or hindered the development under these conditions of the meningitis itself. In civil practice, where cases are not observed so early in the attack, it is exceptional to have to deal with symptoms referable to irritation of the meninges but in which meningococci are present in the blood or even in the cerebrospinal fluid, without, however, being attended by cellular or chemical changes in the cerebrospinal fluid itself. In other words, under the latter circumstances the indications of inflammation are usually present in the meninges, calling for the intraspinal injection of serum. When, however, it happens that no evidence of inflammation is revealed by the lumbar puncture, the question has arisen as to the advisability of making any intraspinal injections of the serum whatsoever.

The reasons for doubt in this instance are two: first, whether any therapeutic benefit will follow; and second, and more important, whether the aseptic meningitis set up by the antimeningococcic serum may favor the local inflammatory attack of the meningococci. The latter point has been raised by Herrick because of the experiments of Flexner and Amoss<sup>10</sup> on the promoting action of an aseptic meningitis induced by serum and other agents on infection in poliomyelitis; but it disregards the important consideration that such an aseptic inflammation is only favoring when a non-immune serum is employed. An immune antipoliomyelitic serum equally sets up an aseptic inflammation, but it also prevents the infection's arising.<sup>10,11</sup> There is therefore no exact analogy to be found be-

1917, lx, 1192, abstracted in *J. Am. Med. Assn.*, 1918, lxx, 498. Baeslack, F. W., Bunce, A. H., Brunelle, G. C., Fleming, J. S., Klugh, G. F., McLean, E. H., and Salomon, A. V., *J. Am. Med. Assn.*, 1918, lxx, 684. Baron and Dumont, *Presse méd.*, 1917, xxv, 394.

<sup>9</sup> Herrick, W. W., *Arch. Int. Med.*, 1918, xxi, 541.

<sup>10</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1914, xx, 249.

<sup>11</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1917, xxv, 525.



tween these experiments on poliomyelitis and the hypothetical problem referred to in meningococcus infection in man.

On the other hand, the newer knowledge on meningococcus infection suggests that experiments be undertaken to determine whether an aseptic meningitis may not promote the passage of meningococci from the blood into the meninges and thus contribute to the inciting of meningococcus inflammation. The monkey is the most suitable animal for these experiments.

In the first place, the monkey can be given meningitis by an intraspinal injection of a suitable culture of the meningococcus. The infection in this case runs a mild or fatal course, depending on the virulence and dose of the culture employed. From the meninges meningococci escape into the general circulation but do not multiply and are quickly suppressed there. When the cultures are injected directly into the blood they do not appear in the meninges in normal monkeys but tend soon to disappear; a meningitis does not arise under these circumstances.

The preceding considerations led us to devise a series of experiments to determine the influence of an aseptic meningitis on the intravenous inoculation of cultures of virulent meningococcus in monkeys. The aseptic inflammation was incited by (a) normal horse serum, (b) normal salt solution, and (c) protargol. The last chemical not only induces an inflammation, but it paralyzes phagocytosis as well, and because of that action intensifies the local action of the meningococcus.<sup>12</sup>

#### EXPERIMENTAL.

The first experiment was arranged to determine the fact of the survival of the meningococcus in the blood and whether it would pass into the cerebrospinal fluid of a normal monkey. Meningococci vary greatly, as stated, in their initial power to set up a severe or fatal meningitis in monkeys on intraspinal injection.<sup>3</sup> When the effect is too slight the virulence of some cultures can be heightened by passage through monkeys or guinea pigs. All monkeys used were *Macacus rhesus*.

<sup>12</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1916, **xxiii**, 683.

*Intraspinal Inoculation.*—An irregular strain of the meningococcus, tending toward the para type, was passed through two monkeys by intraspinal injection, as indicated below, when it acquired a suitable degree of virulence for use. A sheep serum agar slant of a growth 14 hours old was suspended in 2 cc. of isotonic salt solution. Mar. 14, 1918, 4 p.m. Monkey 1 received the entire suspension intraspinally, by lumbar puncture. Mar. 15, 10 a.m. Animal ill. Lumbar puncture, 1 cc. of cloudy fluid containing polymorphonuclear cells and many meningococci. The cocci were mostly extracellular and of typical morphology. A blood culture taken at the same time was negative. Mar. 16, 11 a.m. Animal very ill. Lumbar puncture, 1 cc. of turbid fluid. Microscopic examination showed many pus cells and numerous Gram-negative diplococci, many phagocytized. Two drops of the fluid on sheep serum agar yielded abundant growth. The animal may or may not have recovered, but as the purpose was to recover the meningococcus for subsequent inoculation it was given 2 cc. of antimeningococcic serum intraspinally. Recovery followed.

The culture obtained on Mar. 15 from the spinal fluid was transferred on Mar. 17 to a tube of sheep serum dextrose agar and the growth suspended in the manner described and injected intraspinally at 11.45 on Mar. 18 into Monkey 2. 5.15 p.m. Animal ill. Temperature 39.2°C. Lumbar puncture, 1.5 cc. of turbid fluid under pressure. Microscopically there were large numbers of meningococci and pus cells; few cocci had been phagocytized. The culture yielded abundant growth. Blood culture positive. As the purpose again was to recover the culture, 2 cc. of antimeningococcic serum were injected. The animal gradually recovered.

A third suspension of this meningococcus was prepared in the manner described and injected intraspinally into Monkey 3 on Mar. 20 at 12.10 p.m. 6 hours later the monkey was very ill. Lumbar puncture was performed and antimeningococcic serum administered. Blood culture positive. 3 cc. of turbid fluid were recovered by the lumbar puncture. It contained large numbers of pus cells and meningococci, none intracellular. Mar. 21. Lively. Mar. 22. Completely recovered.

It is obvious from the brief descriptions given that the culture of meningococcus quickly rose in virulence and set up severe symptoms in shorter periods. The following experiments were made with this potent culture.

*Experiment 1. Intravenous Inoculation.*—Mar. 22, 1918, 11 a.m. *Macacus rhesus* given one-half of a suspension of 18 hour growth of culture of meningococcus obtained from Monkey 3 intravenously. 5 p.m. No symptoms. 5 cc. of blood withdrawn from vein. 3 cc. laked and plated. 2 cc. planted in dextrose broth. Former remained sterile; latter gave growth of meningococci.

Lumbar puncture yielded clear fluid devoid of cocci or cells. Mar. 24. No symptoms. Lumbar puncture, no fluid. No symptoms subsequently developed. Apr. 1. Animal lively. Apr. 2. Found dead.

*Autopsy.*—No visceral changes; no cause of death established. Microscopic examination of the central nervous system showed no lesions of any kind. The cause of death can only be conjectured. Possibly it was due to delayed endotoxin intoxication.

This experiment confirms many previously made which are to the effect that an intravenous injection of meningococci into monkeys followed by several lumbar punctures does not set up a meningitis even when the culture is active on meningeal inoculation.

In the next experiments the effects of aseptic meningitis were investigated. In these a strain of parameningococcus recently cultivated from the blood of a case of meningitis in man was employed. It was first tested by intraspinal injection into a *Macacus rhesus* and proved virulent.

*Experiment 2. Intravenous Inoculation Following Intraspinal Horse Serum Meningitis.*—Mar. 24, 1918, 4 p.m. *Macacus rhesus* received 2 cc. of normal horse serum intraspinally. Mar. 25, 10 a.m. Suspension of one-half of sheep serum dextrose agar slant culture in 10 cc. of isotonic salt solution injected intravenously. 5 p.m. 0.5 cc. of blood withdrawn from vein and placed in dextrose broth yielded a growth of meningococcus. Mar. 26, 10 a.m. Lumbar puncture, 0.5 cc. of slightly turbid fluid containing 400 polymorphonuclear cells per c.mm. No meningococci on stained films prepared from centrifuged sediment or in cultures. 5 p.m. same day. 5 cc. of blood taken, laked by addition of two parts of sterile distilled water, centrifuged, and residue smeared on plates. Innumerable colonies of meningococci appeared on the plates. 72 hours after the intravenous inoculation the animal was well; a blood culture was negative, and 0.5 cc. of clear fluid obtained by lumbar puncture was free of meningococci by microscopic and culture test. The monkey remained well.

This experiment was repeated with the same result. The interval between the intraspinal injections of horse serum and the intravenous inoculations of the culture was, however, 40 hours. 1 cc. of blood withdrawn 96 hours after the intravenous injection in dextrose broth yielded a pure culture of meningococcus.

Reference has already been made to the promoting action of an intraspinal injection of normal saline solution in poliomyelitis. As the salt solution sets up an aseptic meningitis of milder degree than



serum it was used in the experiments with meningococcus merely to exclude any protective local action of large numbers of polymorphonuclear leucocytes such as are brought into the meninges by horse serum.

*Experiment 3. Intravenous Inoculation Following Intraspinal Saline Solution.*—The culture of parameningococcus had been passed through two monkeys and recovered from the circulating blood. Apr. 23, 1918, 4 p.m. *Macacus rhesus* received 2 cc. of isotonic salt solution. Apr. 25, 10 a.m. (42 hours after intraspinal injection). Injected intravenously one-half of a 16 hour growth of meningococcus on a sheep serum dextrose agar slant in 10 cc. of isotonic saline solution. Apr. 26, 10 a.m. Lumbar puncture, 1 cc. of clear fluid obtained. Film preparations and culture from bottom of tube after long centrifugation revealed no meningococci. Blood culture of 5 cc. in blood dextrose broth yielded heavy growth of meningococci. Apr. 27, 10 a.m. 5 cc. of blood withdrawn for culture in dextrose broth yielded meningococci. Lumbar puncture, 2 cc. of slightly turbid fluid which was centrifuged at high speed. Film preparations showed polymorphonuclear leucocytes and few round cells but no meningococci. 1 cc. of the fluid containing the sediment planted in dextrose blood broth showed no growth. Apr. 29, 10 a.m. Monkey active and lively. Lumbar puncture, 0.5 cc. of slightly turbid fluid. No meningococci were found on microscopic examination and culture. Blood culture, 5 cc. in dextrose broth negative.

Protargol injected intraspinally not only sets up an inflammation of the meninges, but is antiphagocytic in its action against meningococci. Hence it was employed to promote an intraspinal infection by way of the blood stream.

*Experiment 4. Intravenous Inoculation Following Intraspinal Protargol.*—Mar. 21, 1918, 4 p.m. *Macacus rhesus* received intraspinally 2 cc. of a 0.5 per cent aqueous dilution of protargol. Mar. 22, 10.30 a.m. Injected intravenously one-half of a 16 hour slant culture on sheep serum dextrose agar of the virulent parameningococcus in 10 cc. of isotonic salt solution. 5 p.m. Withdrew 5 cc. of blood, which, after laking with sterile distilled water and centrifuging, were plated on blood agar and yielded a confluent growth of meningococcus. Mar. 23, 10 a.m. Blood culture, 5 cc. plated as before yielded about 200 colonies of meningococci. Lumbar puncture, withdrew 0.5 cc. of slightly turbid fluid. Microscopic examination of film preparation showed a few fragmented, poorly staining polymorphonuclear cells and debris but no meningococci. Culture in blood broth was negative. Mar. 24. Found dead.

*Autopsy.*—Some congestion of brain at the base. Cultures and film preparations from meninges, cortex, lateral ventricles, and base showed no meningococci or other microorganisms. Microscopic sections showed slight meningeal inflammation as indicated by a collection of leucocytes probably due to the protargol injection.



This experiment was repeated so that the observations extended over a somewhat longer period of time. Because of the early disappearance of the meningococci from the blood after injection several intravenous injections of living meningococci were made at intervals to prolong the period of the meningococcemia.

*Experiment 5. Multiple Intravenous Inoculations Following Intraspinal Injection of Protargol.*—Mar. 27, 1918, 4 p.m. *Macacus rhesus* injected intraspinally with 2 cc. of a 0.5 per cent aqueous dilution of protargol. Mar. 28, 11 a.m. Injected intravenously with one-half of a 16 hour growth on sheep serum agar of a virulent parameningococcus. 3 p.m. Blood culture positive; total leucocytes 10,200. Mar. 29, 11 a.m. Blood culture positive; lumbar puncture yielded 2 cc. of slightly turbid fluid containing fragmented cells but no meningococci. Cultures remained sterile. Mar. 30, 11 a.m. Lumbar puncture yielded 2 cc. of fluid; no meningococci found on culture or microscopic examination. Blood culture (5 cc.) positive; total leucocytes 7,600. Mar. 31, 11 a.m. Lumbar puncture, 0.5 cc. of fluid; no meningococci found by culture or microscopic examination. Blood culture (5 cc.) positive. Apr. 1, 11 a.m. Lumbar puncture, 3 cc. of fluid. No meningococci found by culture or microscopic examination. Blood culture (8 cc. in broth) negative.

The animal never showed any symptoms of meningitis and was allowed to rest 16 days to develop a partial immunity, for two purposes: first, to ascertain whether subsequent injections under conditions of partial immunity would allow meningeal invasion by the clumping of the organisms (*in vivo* agglutination) as sometimes happens in dogs injected with living pneumococci;<sup>13</sup> and second, to ascertain whether the organisms introduced by spinal injection would be found in the blood stream in the highly active immune animal.

Apr. 13. Injected intravenously one-half of a 20 hour growth of parameningococcus on slant serum agar in 10 cc. of isotonic salt solution. Apr. 20. Injected intravenously three-quarters of a 16 hour culture of parameningococcus. Apr. 27. Injected the entire culture. The animal remained well. Before injection serum agglutinated the homologous parameningococcus completely in a dilution of 1:1,200 and other parameningococci in 1:1,000. May 2, 5 p.m. Injected intraspinally in 5 cc. of isotonic salt solution entire 8 hour growth of virulent parameningococci on slant serum agar. May 3, 11 a.m. Total white blood cells 24,200. 5 cc. of blood withdrawn for culture yielded meningococci. Lumbar puncture, 0.1 cc. of slightly turbid fluid from which large numbers of meningococci were grown. May 4, 11 a.m. Growth obtained from 1.5 cc. of blood withdrawn for culture. Total white blood cells 40,400. Lumbar puncture, 2 cc. of turbid fluid from which cultures were positive. Microscopic exami-

<sup>13</sup> Bull, C. G., *J. Exp. Med.*, 1916, xxiv, 7.

nation showed numerous Gram-negative diplococci intracellular; few extracellular. May 5. Total white blood cells 12,400. Blood culture (5 cc.) negative. Lumbar puncture, 3 cc. of slightly turbid fluid containing many phagocytosed Gram-negative diplococci, none extracellular. Cultures negative. May 6. Total white blood cells 12,000. Blood culture (5 cc.) negative. Lumbar puncture, 3 cc. of slightly turbid fluid. Culture negative. No organisms found on microscopic examination.

In this experiment the living virulent meningococci circulated in the blood for at least 72 hours during an aseptic inflammation of the meninges without passing through and causing meningitis. The monkey was actively immunized by intravenous injections so that the serum agglutinated the meningococcus in a dilution of 1:1,200. The intraspinal injection of meningococci was followed by meningococcemia lasting 48 hours, showing that whereas the passage of these organisms from the blood to the cerebrospinal fluid could not be brought about even under conditions promoting interchange, ready passage in the opposite direction from the meninges to the blood was accomplished when antibodies were circulating in the blood.

### *Experiments with Rabbits.*

Austrian<sup>14</sup> reports that he was able to observe localization of the meningococcus in the meninges and typical fatal meningitis after intravenous injection in three rabbits out of twenty which had received a previous intraspinal injection of normal rabbit serum. In the rabbits which received no preparatory intraspinal injections localization did not occur.

However, it is stated in his typical protocol that after the intraspinal injection of normal rabbit serum "injury of the cord caused twitching of the tail and spastic palsy of the left hind leg, and dyspnoea, coma and ataxia developed." It is possible, therefore, that mechanical rather than chemical injury made possible the passage of the microorganisms from the blood to the spinal fluid. In order to test the validity of this view two series of rabbits were injected intraspinally with normal rabbit serum. In the first series great care was taken to avoid mechanical injury to the cord, while in the second series

<sup>14</sup> Austrian, C. R., *Bull. Johns Hopkins Hosp.*, 1918, **xxix**, 183.

the needle was introduced so that spastic paralysis followed the procedure. The animals of both series subsequently received intravenous injections of living meningococci.

*Series I.*—Five rabbits weighing about 2 kilos received intraspinally 1.5 cc. of normal rabbit serum. Half an hour later there were injected intravenously in 10 cc. of saline solution the growths from two 16 hour cultures on slant serum agar of a virulent irregular meningococcus. Rabbit 1 was etherized 4 hours later, No. 2 died in 6 hours, No. 3 was etherized at 8 hours, and Nos. 4 and 5 at 24 hours. Cultures and film preparations from the cord, cortex, pons, base of brain, and heart's blood showed no meningococci.

*Series II.*—Rabbit 6, weight 2.2 kilos, injected intraspinally with 1.5 cc. of normal rabbit serum on Oct. 15, 1918, 3 p.m. 3.05 p.m. Spastic; paralysis of both hind legs. Oct. 16, 10 a.m. No paralysis. Injected intravenously, in 10 cc. of isotonic salt solution, the 16 hour growth from two serum agar slants of a virulent irregular meningococcus. 4 p.m. No symptoms. Etherized and autopsied. Lumbar portion of cord hemorrhagic. Film preparations of cord and base of brain showed polymorphonuclear leucocytes and a few Gram-negative diplococci, none on cortex. Cultures from heart's blood and base of brain yielded irregular meningococci.

Rabbit 7, weight 2 kilos, received intraspinally 1.5 cc. of normal rabbit serum on Oct. 15, 1918, 3 p.m. 3.30 p.m. Spastic paralysis of left hind leg. Oct. 16, 10 a.m. Paralysis persisted. Injected intravenously in 10 cc. of isotonic salt solution 16 hour growth from two serum agar slants of a virulent irregular meningococcus. Oct. 17, 10 a.m. Paralysis persisted. No other symptoms. Etherized.

*Autopsy.*—Small hemorrhage in lumbar portion of cord. The blood vessels of the pia and dura were congested. Spinal fluid was turbid. Film preparations from spinal meninges, pons, and base of brain showed considerable number of polymorphonuclear leucocytes and many Gram-negative extracellular diplococci. Cultures from the spinal fluid, base of brain, and pons yielded meningococci, but the cultures from heart's blood were negative.

These experiments indicate that even under the conditions of congestion and chemical inflammation the meningococci do not pass from the blood to the spinal fluid in the rabbit unless a break in the continuity of the tissues occurs. The mechanical injury allows passage and localization of the meningococci. The results of the negative experiments in the monkey (Table I) correspond to those obtained in Series I with rabbits.

TABLE I.

*Results of Intravenous Injections into Monkeys of Living Virulent Meningococci Following Intraspinal Injections of Substances Producing Chemical Inflammation.*

Experiment No.	Date.	Previous intraspinal injection.	Intravenous injection.	Last positive blood culture obtained at.	Result
1	1918 Mar. 22	None.	One-half 16 hr. growth on serum agar slant.	24th hr.	No localization in meninges.
2	" 25	2 cc. of normal horse serum.	" "	24th "	" "
3	Apr. 24	2 cc. of normal horse serum.	" "	96th "	" "
4	" 24	2 cc. of isotonic salt solution.	" "	48th "	" "
5	Mar. 22	2 cc. of 0.5 per cent aqueous suspension of protargol.	" "	24th "	" "
6	" 28	2 cc. of 0.5 per cent aqueous suspension of protargol.	" "	72nd "	" "

## DISCUSSION.

In discussing the several experiments described in this paper it will be well first to formulate the problem which presented itself for solution and then the means at hand to be employed for the purpose.

There has been no convincing finding either in man or in experimental animals to show how the meningococcus reaches the meninges. Two possibilities are obvious: lymphatic and hematogenous extension. The first would carry the meningococci directly from the nasopharynx to the base of the brain; the other indirectly, by way of the general circulation, to the subarachnoid space.

As long as cases of frank meningitis only came under observation the meningococci were usually found with ease in the turbid cerebrospinal fluid and possibly also in the blood. Flexner<sup>3</sup> showed in his experiments on the intraspinal inoculation of meningococci in monkeys that passage of the organism takes place from the subarachnoid space into the blood. Hence the mere finding of the



meningococci in the circulating blood in cases of meningitis in man could not be taken to indicate one way or the other their relation to the meningeal infection.

In one of the experiments recorded here (Experiment 5) intraspinal injection of meningococci into the actively immune monkey was followed by the appearance of these organisms in the blood, where they persisted for 48 hours. The serum at this time agglutinated the particular strain of meningococci in a dilution of 1:1,200. This indicates that passage is far more easily accomplished by the meningococci in one direction, from the spinal fluid to the blood, than in the opposite direction, blood to spinal fluid.

The study of very early cases of infection in military establishments has thrown new light on the mechanism of meningococcus infection. For leaving aside the rare cases of meningococcemia without meningeal localization, it appeared that not infrequently cases would come under observation said to show no inflammatory changes in the meninges, as determined by a study of the spinal fluid, but with severe general symptoms of infection (including subcutaneous hemorrhages) in which meningococci were cultivable from the circulating blood. If these cases were not promptly treated and arrested by specific serum therapy, a meningitis is stated soon to develop. It may be that the presence of the meningococcus in the blood precedes their occurrence in the meninges or that the organism, on entering the cranial cavity and the blood simultaneously, is more readily recovered from the blood stream, or lastly that it is established in the upper portions of the central nervous system and overflows into the blood stream before evidences appear in the spinal fluid withdrawn at the remote and lower parts of the central nervous system.

In endeavoring to find a solution of the problem presented by experiments two points were kept in mind: first, that of a possible primary blood infection; and second, a promoting effect of the intraspinal injection of a serum on the localizations of the meningococcus in the meninges.

As regards the possibility of obtaining a decisive result by experiments on monkeys, the first point to be taken into account is the relative insusceptibility of the monkey to infection with the meningococcus. This degree of resistance is so great that Flexner's questions

whether, after an intraspinal injection, in which a fatal meningitis is set up, any actual multiplication of the microorganism takes place. He thinks it not impossible that from the beginning the injected micrococci are killed off, and the final fatal effect is caused by intoxication.

There can therefore be no very close analogy between the conditions experimentally induced and those existing in man when meningococcus infection arises. The experiments presented, however, are entirely clear on one point; namely, that in the monkey the meningococcus cannot be made to pass from the circulating blood, in which it is proved still to be surviving, to the aseptically inflamed meninges in such a way as to be detected there either by microscopic examination or in cultures. In other words, as far as the monkey is concerned the production of an aseptic meningitis is wholly without effect on the fate of meningococci injected into the blood stream.

The experiments are therefore in strong contrast to those made with the poliomyelitic virus, just as the great activity of the poliomyelitic virus when implanted on the nasal mucosa is in striking contrast to the innocuousness of the meningococcus applied to the same membrane. The experiments described in this paper emphasize, therefore, the unsuitability of the lower monkeys to solve the problem of the mode of meningococcus infections in man. And as far as they go they tend to show that an aseptic meningitis does not predispose the meninges to infection from the blood stream. Since in man the serum injected intraspinally is not normal but immune it can be presumed that the increased permeability of the meninges and choroid plexus induced by the chemical inflammation set up would be more than offset by the immunity principles introduced into the subarachnoid space along with the serum.

#### CONCLUSIONS.

The lower monkeys as represented by *Macacus rhesus* are resistant to a high degree to infection with cultures of the meningococcus introduced into the general blood.

The lower monkeys are less resistant to infection when the meningococcus cultures are injected directly into the subarachnoid space by lumbar puncture.

Relatively virulent cultures, which have been passed through several monkeys, acquire the power of surviving in the circulating blood of the monkeys for a maximum period of about 72 hours. Nothing has, however, been observed to indicate that the injected meningococci actually multiply in the blood.

It has not been found possible to direct the meningococci circulating in the blood into the cerebrospinal meninges of monkeys. In this effort an aseptic meningitis was induced by injecting horse serum, saline solution, or protargol into the subarachnoid space preceding the introduction of the meningococci into the blood.

In rabbits the meningococci were able to pass into the spinal fluid from the blood when a physical break in the continuity was made; however, under the conditions of chemical inflammation of the meninges the rabbit reacted just as the monkeys, and the organisms did not pass.

Because of the high insusceptibility of the monkey to infection with meningococcus, it is not believed that the experiments throw any new light on the mode of invasion of the body in man by that microorganism.

The experiments do not lend any support to the notion that an intraspinal injection of the antimeningococcus serum, early in the course of invasion of meningococcus in man, and possibly at a period at which the meninges do not yet show evidences of inflammation, favors its diversion from the blood stream into the subarachnoid space.

## GROWTH ACCESSORY SUBSTANCES FOR PATHOGENIC BACTERIA IN ANIMAL TISSUES.

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The subject of vitamins has of late occupied the attention of the biologist and biological chemist. The nature of these substances is still a matter of speculation, but the majority of workers accepts the distinction proposed by McCollum and Davis. These authors recognize two classes of substances, the fat-soluble A and the water-soluble B. A more precise definition has not been possible, because of the failure thus far to isolate and identify the respective compounds. Their presence can be detected only by the effect they produce on the growing organism, usually the white rat. By noting the effect of the addition of various substances to a balanced vitamin-free diet, one can obtain a rough indication of the concentration of food accessory substances in the added material.

The subject of the relation of vitamins to bacteria has recently been approached from two directions. One group of workers studied the bacteria with a view to discovering the source of vitamins. Since plants (Bottomley) and animals (McCollum and Simmonds, Osborne and Mendel, Loeb and Northrop) cannot synthesize these substances, their origin must naturally be looked for among the lower microorganisms. Mockenridge has indeed shown that certain soil bacteria are capable of elaborating these substances, although *Bacillus radicicola*, actively synthetic and of simple food requirements, cannot do so. Pacini and Russell report experiments indicating that the typhoid bacillus grown in Uschinsky's medium produces vitamins. The other group of workers was more concerned in determining the relation of vitamins to the cultivation of the more delicate pathogenic bacteria. Cole and Lloyd demonstrated their importance for the growth of the gonococcus, while Lloyd and Gorden, Hine, and



Flack showed that they were essential also for the growth of the meningococcus. Evidently neither of the organisms can multiply actively in the absence of these substances.

The object of this paper is to report experiments bearing on (a) the effect of vitamins on the growth of a number of organisms pathogenic for man; (b) the distribution of these substances in animal tissues; and (c) the relative significance of the fat-soluble A and water-soluble B in the cultivation of these microorganisms. At present there are few data bearing on these questions in relation to bacteria

#### EXPERIMENTAL.

##### *Methods.*

In the course of the investigation beef heart, goat blood, rabbit and cat tissues, and human secretions were used. Unless otherwise stated, the method of extraction was always the same. The tissue or organ was obtained as free from blood as possible. The animals were first exsanguinated and the tissues were washed with saline solution, free from visible traces of blood. The material was then weighed, macerated into small bits, suspended in nine times its weight of saline solution, shaken thoroughly, and placed in the ice box over night. The following day the extract was centrifugalized and filtered through a Berkefeld candle. After testing for sterility the extracts were ready for use.

The effect of these extracts was tested by adding graded amounts to nutrient broth or agar, or to phosphate peptone agar and inoculating with small amounts of culture suspensions. The tubes were incubated at 37°C. and observations were made daily for several days. Control tubes without extract were always used.

The following organisms were tested:<sup>1</sup> gonococcus, meningococcus (one para, one regular), Pneumococcus Type I, *Streptococcus hæmolyticus*, *Bacillus diphtheriæ*, *Bacillus pertussis*, *Bacillus influenzae*.

<sup>1</sup> Cultures of *B. typhosus* and of the Shiga and Flexner varieties of *B. dysenteriae* were included in the early experiments, but as no differences were observed between the growth on the control media and on those containing the extracts, they were later excluded.

To avoid the error that would result from carrying over vitamins with the bacterial mass, small inocula were used. Usually 0.1 cc. of a 24 hour broth culture was suspended into 5 cc. of peptone or saline solution, and of this suspension 0.1 cc. was used for inoculating broth tubes and a 4 mm. loop for the agar slants. Before making the suspensions the cultures were first diluted to a turbidity corresponding to a 24 hour broth culture of the pneumococcus.

### Results.

Most of the experiments reported below were repeated from one to four times. Either broth or agar media or both were used. As a rule (when the size of the inoculum was just right), the effect of the extract was manifested both by the earlier appearance and greater abundance of the growth. The extracts, with few exceptions, influenced favorably the growth of all the bacteria studied, and the effect varied with the amount of extract added.

*Effect of Beef Heart Extract.*—The following is a typical experiment with beef heart extract. The extract was prepared from fresh beef heart in the manner outlined, and varying amounts were added to tubes containing 5 cc. of nutrient broth. The results are shown in Table I. Similar results were obtained with the use of agar as a base.

TABLE I.  
*Effect of Heart Extract on the Growth of Bacteria.*

Culture.	Amount of extract.									
	1.0 cc.		0.1 cc.		0.01 cc.		0.001 cc.		0.0 cc.	
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.
Meningococcus.....	+	+	+	+	—	±	—	—	—	—
Pneumococcus.....	+	+	+	+	+	+	—	+	—	+
Streptococcus.....	+	+	—	+	—	—	—	—	—	—
<i>B. diptheria</i> .....	±	+	—	+	—	+	—	+	—	+
<i>B. pertussis</i> .....	+	+	+	+	—	+	—	+	—	+
<i>B. influenza</i> †.....	—	+	—	+	—	—	—	—	—	—

\* The plus signs indicate the presence and relative abundance of growth.

† Inoculations of the influenza bacillus were made with a 4 mm. loop direct from blood broth cultures; small amounts of blood were thus carried over, but not enough to permit growth in plain broth.

*Effect of Extracts of Various Organs and Tissues of the Rabbit and Cat.*—A uniform procedure was employed in the following experiments. The animal was etherized, exsanguinated, and the organs or tissues were removed aseptically to sterile Petri dishes. The extracts were made as described. With stomach or intestinal mucosa the organs were first washed free of fecal matter, the mucosa was then scraped off with a scalpel and suspended in nine times its weight of saline solution. Extracts from tissues of four rabbits and one cat were tested in the course of the experiments. The method of testing was the same as that described above.

The results were strikingly uniform. When 1 cc. of the extracts was added, no differences in effect were discernible. Extracts of all the tissues tested had a decidedly stimulating action on the growth of the various test organisms. However, when smaller amounts of the extract were added, certain fairly constant differences were observed. Extracts of liver and stomach mucosa were more effective than those of other tissues, while those of brain and muscle were less so. The influence of other tissue extracts was practically the same. They had somewhat different effects on different bacteria, but that may have been due to the error in technique which is considerable.

Table II gives the results obtained in one series of tests. It is difficult to summarize the data. Attention can only be called to some of the points which cannot be tabulated. The differences between the tubes containing larger and those containing smaller amounts of the extract were always more marked after 24 than after 48 hours. Not only were the colonies on the slants of the former more numerous, but they were, as a rule, larger. The pneumococcus showed a strikingly different behavior on spleen extract agar than on the others. On the former medium autolysis usually occurred 24 to 48 hours after it had appeared in all the other tubes. *Bacillus influenzae* failed to grow on any of the media until a trace of blood was added. When a drop of a saline suspension of red cells was added to the slant, the growth was practically the same in all the tubes. The results with the gonococci were extremely variable. Even under favorable conditions growth is much slower than that of any of the organisms tested. As a rule, 3 to 4 days elapse before fair sized colonies appear. By that time the tubes are apt to be dry,

and moisture is essential for their growth. They did, however, grow as well on agar containing 0.1 cc. of the extracts as on sheep serum glucose agar.

TABLE II.

*Effect of Extracts of Rabbit Tissues on the Growth of Bacteria.*

Culture.	Amount of extract.	Extract.								
		Liver.	Spleen.	Kidney.	Heart.	Lung.	Muscle.	Brain	Testes.	Stomach.
	cc.									
Streptococcus....	0.1	++	++	++	+	+	+	+	+	++
	0.01	=	=	=	=	=	=	=	=	+
	0.0	=	=							
Pneumococcus....	0.1	+++	+++	+++	+++	++	++	++	+++	+++
	0.01	++	++	++	++	++	++	++	++	+++
	0.0	+	+							
<i>B. diphtheria</i> .....	0.1	++	++	++	++	++	+	=	=	++
	0.01	+	=	++	=	=	=	Tr.	=	++
	0.0	-	-							
Gonococcus.....	0.1	+	+	++	+	+	+	+	+	++
	0.01	+	+	++	+	+	+	+	+	++
	0.0	-	-							
Meningococcus...	0.1	++	++	++	++	++	+	=	++	+++
	0.01	++	+	+	++	++	+	Tr.	++	+++
	0.0	-	-							

*Growth Accessory Substances in Extract of Mucosa of Various Organs.*

—The interesting fact brought out by the previous experiments was that extract of stomach mucosa stimulated the growth of bacteria more actively than those of other tissues. It was of interest to note whether extracts of other mucosa would have a similar effect. It was also anticipated that these extracts might show some specific selective action. Tests were made with nasopharyngeal, stomach, intestine, and genitourinary mucosa. The usual method of extraction and testing was employed. The results showed clearly that extracts of all the mucosa tested had a favorable effect on growth. No



conclusion could be drawn from these experiments regarding their specificity, because of the large element of error involved in the technique.

*Growth Accessory Substances in Cat Tissues.*—The object of these tests was to determine whether there was any marked difference in the distribution of the growth accessory substances in different animals. The extracts were made and tested in the usual manner. The only difference in technique was the use of peptone phosphate agar and peptone phosphate broth in place of the beef infusion medium. The former furnished a vitamine-free base, and the effects of the extracts were brought out more strikingly than when infusion media

TABLE III.  
*Effect of Extracts of Cat Tissues on the Growth of Bacteria.*

Culture.	Control.	Extract (0.1 cc. to tube).							
		Liver.	Spleen.	Kidney.	Heart.	Lung.	Brain.	Stomach.	Intestines.
<i>Streptococcus</i> .....	—	++	++	++	++	++	+	++	++
<i>Pneumococcus</i> .....	—	++	++	++	++	+	++	++	++
<i>B. diphtheriæ</i> .....	—	++	++	++	++	++	++	++	++
<i>Meningococcus</i> .....	—	++	++	++	++	++	++	+++	++
<i>B. pertussis</i> .....	—	+	+	#	+	#	#	#	+

were used. Otherwise the results were essentially the same as those obtained with rabbit tissue extracts. As was previously noted, the autolysis of the pneumococcus occurred later on liver and spleen extract media than on the others. The results are shown in Table III.

*Effect of Tissue Extracts on Lag.*—The phenomenon of lag is of practical as well as theoretical interest. It has been studied extensively by a number of workers, among them Müller, Penfold, and Chesney. The most carefully worked out theory explaining this phenomenon is that advanced by Chesney. He believes that lag represents the time necessary for the bacterial cells to recover from injury.

It seemed of interest to determine the effect of the extracts on the lag phase, or, in other words, the influence of growth-stimulating substances on the speed of cell recuperation. Beef heart and rabbit tissue extracts were used. The streptococcus and pneumococcus served as the test cultures, because of the ease with which they can

TABLE IV.  
*Effect of Beef Heart Extract on Bacterial Lag.*

Test culture, <i>Streptococcus hemolyticus</i> .				
Platings.	Time interval.	No. of colonies per cc. of broth.		
		Extract, 1 cc.	Extract, 0.1 cc.	Broth.
	<i>hrs.</i>			
1	0	2,300	3,300	1,300
2	1	3,800	3,900	2,500
3	3	32,000	11,000	1,400
4	5	230,000	14,000	>100
5'	6	320,000	43,000	>100
	24	Heavy turbidity.	Sterile.	Sterile.

TABLE V.  
*Effect of Beef Heart Extract on Bacterial Lag.*

Test culture, pneumococcus.				
Platings.	Time interval.	No. of colonies per cc. of broth.		
		Extract, 1 cc.	Extract 0.1 cc.	Broth.
	<i>hrs.</i>			
1	0	3,200	3,000	3,200
2	1	3,700	3,200	4,000
3	3	4,000	4,000	4,100
4	5	4,600	4,300	1,300
5	6	15,000	8,000	>1,000
	24	Good turbidity.	Sterile.	Sterile.

be manipulated. To tubes containing 5 cc. of broth were added graded amounts of the extract. Duplicate tubes of broth and extract broth were inoculated with the same amount of a culture suspension. Glucose agar plates were made immediately and at stated

intervals. The plates were incubated at 37°C. for 24 hours, and the number of colonies was counted. The figures in the tables give the average number of colonies per cubic centimeter of broth.

The results are shown in Tables IV, V, and VI. These tables show clearly that beef heart and rabbit tissue extracts have a decided influence on bacterial lag, and, moreover, that the effect varies with the amount of extract added. The smaller the amount of extract, the longer is the lag phase; and when the concentration of the accessory substances is too low, the results are the same as in the broth controls.

Although this is not a study of lag, the bearing of these experiments

TABLE VI.  
*Effect of Rabbit Tissue Extracts on Bacterial Lag.*

Test culture, pneumococcus.							
Plating interval.	No. of colonies per cc. of culture at time of plating						Broth C.*
	Stomach extract.		Heart extract.		Muscle extract.		
	0.5 cc.	0.1 cc.	0.5 cc.	0.1 cc.	0.5 cc.	0.1 cc.	
<i>hrs.</i>							
0	15,000	15,000	18,000	17,000	17,000	17,000	18,000
2	22,000	15,000	17,000	17,000	18,000	17,000	18,000
4	38,000	26,000	60,000	25,000	36,000	9,500	14,000
5	71,000	53,000	73,000	46,000	71,000	7,000	5,000
6	180,000	90,000	148,000	87,000	210,000	4,500	7,000
24	+++	+++	+++	+++	+++	Sterile.	Sterile.

\* Two broth controls were run with the same results.

on the explanation of this phenomenon should be noted. The fact that even under favorable conditions there is a quiescent period before active growth commences, confirms Chesney's assumption of recuperative adaptation. It appears, however, that recuperation is not possible unless some substances are present which favor the active initiation of growth. Ordinarily this is apparently supplied by the disintegrating cells. This interpretation would account for the need of heavy inocula for successful transplants of cultures.

Besides throwing some light on the lag phenomenon, these experiments confirm the fact that the tissue extracts contain some

substances favorable to bacterial growth. All of them in certain concentrations convert an unfavorable substrate where growth does not occur into a favorable one. This effect is produced in the case of rabbit heart and stomach extracts by the addition of only 0.1 cc., while in the case of muscle extract of the same animal a larger amount must be added to obtain that result. The influence of the extract must, therefore, depend on the concentration of some substance or substances in them. Whatever the nature of these substances, it is of interest to note that they are present in lesser concentration in extract of muscle than in that of heart or stomach mucosa.

*Effect of Nasal Secretions on Growth of Bacteria.*—Shearer has reported experiments showing the stimulating action of nasal secretions on the growth of the meningococcus. It seemed worth while, in view of the results recorded above, to confirm and possibly extend these observations. The tests were limited to nasal washings and saliva.

The first tests were made with nasal secretions. These were mainly confirmatory of the work of Shearer. The secretions were obtained by blowing the nose into strips of sterile gauze. These were put into saline solution or alcohol, and kept for 3 days. The saline extract was divided into two parts, one of which was filtered through a Berkefeld candle, while the other was autoclaved. The alcoholic extract was desiccated to dryness, and then taken up in saline solution.

These extracts were added in 1 cc. and 0.5 cc. amounts to tubes containing 10 cc. of glucose agar and 1 cc. of sheep serum, and plates were poured. The plates were sown with broth suspensions of meningococci; blood and serum plates served as controls. Both saline extracts were superior to the alcoholic extract, though the latter improved the growth as compared with the serum plate. The filtered extract was more effective than the heated one. In no case was the growth so good as on the blood agar plate. The results showed clearly that the nasal mucus contains some substance which favors growth.

The subsequent experiments were modified with a view to determining whether pathogenic bacteria were capable of growing in the



secretions, and, if so, whether there was any difference in their ability to do so. For this purpose saline washings of the nose of apparently normal individuals as well as saliva were used; 50 cc. of warm saline solution were used for each washing. These were collected in sterile bottles, filtered through a Berkefeld candle, tested for sterility, and tubed in 2 cc. amounts in small test-tubes. The saliva was collected in the course of the day, filtered through a Berkefeld candle, and tubed in the same way.

*Growth in Nasal Washings.*—The nasal washings of five adult individuals and one rabbit were used. The washings were all ob-

TABLE VII.

*Growth of Pathogenic Bacteria in Nasal Washings.*

Source of washings.	Culture.															
	Meningococcus.				Gonococcus.		Streptococcus.		Pneumococcus.		<i>B. diptheriæ.</i>		<i>B. pertussis.</i>		<i>B. influenza.</i>	
	Para.		Normal.													
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.
Rabbit.	—	—	—	—	—	—	+	+	+	+	—	—	+	+	—	—
W.	+	+	+	+	—	±	+	+	+	+	—	—	+	+	+	+
D.	—	—	—	—	—	Tr.	+	+	+	+	—	—	—	—	—	—
Z.	+	+	—	—	—	Contaminated.	—	—	—	—	±	±	—	—	+	+
K.	+	+	+	+	—	“	+	+	+	+	—	—	+	+	+	+
T.	—	—	—	—	—	±	+	+	+	+	+	+	+	+	—	—
Saliva K.	—	—	—	—	—	—	—	—	—	—	±	+	—	—	—	—
“ Z.	—	—	—	—	—	—	—	—	—	—	±	+	—	—	—	—

tained on the same day and treated in the same manner. The inoculations were made by diluting 0.1 cc. of a 6 hour broth culture in 5 cc. of broth and adding 0.1 cc. of the suspension to each tube. Growth was determined by turbidity and checked by means of Gram-stained films. The results are shown in Table VII.

The washings were made as nearly as possible in the same manner. The rest of the procedure was identical. The same cultures were used for inoculating all the tubes. Yet there were marked individual and specific differences. A given organism grew in the washings of one individual and not in those of another. Chemical tests revealed no differences in the washings. They were all slightly acid

when fresh, but reverted to alkalinity on standing (probably due to a loss of carbon dioxide). No amino nitrogen and only a trace of nitrogen could be detected in 5 cc. of the washings. Reduction tests for glucose were also negative. Nevertheless they offered a favorable substrate for growth while saline controls were negative. No growth appeared in the saliva tubes. Evidently the nasal secretions of some individuals contain substances which actively stimulate growth, while saliva does not.

*Relative Effect of Saline and Ether Extracts.*—To ascertain the relative importance of the water-soluble and fat-soluble fractions, com-

TABLE VIII.  
*Growth Accessories in Ether and Saline Extracts.*

Extract.	Culture.					
	Streptococcus.	Pneumococcus.	<i>B. diptheria</i> .	Meningococcus Normal.	<i>B. pertussis</i> .	<i>B. Influenza</i> .
Saline.....	+	+	++	+	±	—
Ether.....	±	—	±	±	—	—
Mixture.....	+	+	+	+	±	—
Peptone.....	—	—	±	—	—	—

parative tests were made with saline and ether extracts. Blood clot was used for this experiment; it is rich in vitamins and has a markedly stimulating effect on the growth of bacteria. Equal portions of blood clot, from which the serum had been completely removed by centrifugalization, were weighed out. One portion was suspended in ten volumes of saline solution, the other in ten volumes of alcohol ether (1:3). The alcohol was added first to desiccate the clot and the ether was then added. The saline extract was filtered through a Berkefeld candle. The ether extract was decanted, evaporated nearly to dryness under suction, and the residue taken up in an amount of saline solution equal to the original volume.

Media containing these extracts were then prepared. This time a peptone phosphate solution was used as a base in place of nutrient broth to eliminate all traces of vitamins from other sources. 1 cc. of the respective extracts, as well as a mixture of the two, was added to tubes containing 5 cc. of the peptone solution. These were inoculated in the usual manner and incubated at 37°C.

The results are tabulated in Table VIII. It is evident that the saline extract contains some substances which favor the growth of bacteria, while the ether extract is practically devoid of them. Moreover, inoculations of the various organisms made into the extracts directly failed to give growth. Neither the extract alone nor the peptone alone was capable of supporting growth. The two together constituted a favorable medium. Similar results were obtained with saline and ether extracts of beef heart.

*Nature of the Growth Accessory Substances in These Extracts.*—The question naturally arises: To what is the favorable effect of these extracts due? Is the improvement of the medium attributable to the addition of food accessory substances or merely to an enrichment in its nutritive quality? This question is naturally difficult to answer, but some experiments have been made which indicate that we are dealing with a vitamine stimulation.

1. If the effect of the extracts is the result of a high content of nutritive substances, bacteria should grow abundantly in them. Tests showed that although moderate growth occurred in the concentrated extracts, none appeared when the extract was diluted five times with saline solution.

2. It has been assumed that the addition of serum enhances the value of the medium, because of the "native" protein. The proteins of the extract were precipitated with alcohol and removed by centrifugalization. The supernatant fluid freed from the alcohol by evaporation under vacuum had the same effect as the original extract.

3. There is some contradiction in the literature regarding the heat stability of vitamins, but it is generally agreed that high temperatures partially or completely destroy these substances (Chick and Hume). On the other hand, amino-acids or carbohydrates, the essential nutritive substances for bacteria, are not affected by heat.

Extracts were prepared in different ways, by employing heat, and the effect on growth was compared with those prepared at the ice box temperature. Lean fresh beef heart was used. Equal weights were macerated and taken up with nine volumes of saline solution. These suspensions were treated respectively as follows: (1) kept in ice box over night and steamed in an Arnold sterilizer for 1 hour;

(2) kept in ice box over night and filtered through a Berkefeld candle; (3) kept at 55°C. over night and heated in an Arnold sterilizer for 1 hour; (4) kept at 55°C. over night and filtered through a Berkefeld candle; (5) extracted by boiling for 1 hour and sterilized in an Arnold sterilizer for 1 hour; (6) extracted by boiling for 1 hour.

The relative effect of these six extracts was tested in the usual way. The results with the agar slants were more striking than those obtained with the broth and are shown in Table IX.

TABLE IX.

*Effect of Heat on the Concentration of Substances Favoring the Growth of Bacteria.*

Agar slants. Relative abundance of growth after 24 hrs. at 37°C.

Extract.	Culture.					
	Strepto- coccus.	Pneumo- coccus.	<i>B. diphtheriæ.</i>	Meningococcus.		<i>B. pertus- sis.</i>
				Normal.	Para.	
Ice-Arnold.....	++	++	—	±	—	±
Ice-filter .....	++++	+++	++	+++	++	+
55°C.-Arnold.....	+	+	—	±	—	±
55°C.-filter.....	++++	++	++	+++	++	+
Boiling-Arnold.....	+	+	—	±	—	±
Boiling .....	++	+	±	±	—	±
Plain agar .....	+	+	1 colony.	±	—	—

The results shown in Table IX indicate clearly that heat destroys to some extent the substances which favor bacterial growth. Extracts prepared at ice box temperature or at 55°C. are superior to those obtained by heating, and the longer the heating, the less effective are the extracts. The fact that these substances are heat-labile supports the view that they belong to the class of growth accessory substances.

## SUMMARY.

The growth of all the pathogenic bacteria studied was favorably influenced by the addition of small amounts of tissue extracts.

Beef heart, rabbit and cat tissues, and human nasal secretions contain substances favorable to the growth of the organisms tested. The mucosa of different organs, spleen, liver, and kidney, are relatively rich in these substances, while muscle is relatively poor. The favor-



able effect of the extracts is manifested by an enhancement of growth and a reduction of lag.

The water-soluble substances are apparently the ones essential for bacterial development; the ether extract has no effect on growth.

Experiments are reported which indicate that the substances in question belong to the class of so-called vitamins.

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## SYNTHESES IN THE CINCHONA SERIES. I.

### THE SIMPLER CINCHONA ALKALOIDS AND THEIR DIHYDRO DERIVATIVES.

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Interest in the cinchona alkaloids as material for chemotherapeutic study has recently been revived by the remarkable specificity for the pneumococcus shown by ethylhydrocupreine.<sup>1</sup> The present paper is part of an investigation undertaken to test the possibilities for synthetic work in this field and deals with a number of the cinchona alkaloids, their reduction products, and certain synthetic homologs of the latter.

The cinchonine, cinchonidine, quinine, and quinidine used were purchased in the open market, and the constants obtained in the case of their monohydrochlorides are given below for comparisons with the corresponding data for the other alkaloids considered.

The reduction for the four alkaloids to hydrocinchonine, hydrocinchonidine, hydroquinine, and hydroquinidine was very easily accomplished according to German patent 252,136, method 2, using palladium chloride in dilute sulfuric acid solution. Details of the method are given below for the case of hydroquinine. The properties of the hydrogenated alkaloids agreed with those recorded in the literature for the naturally occurring substances, and a direct comparison was made in the case of hydrocinchonine with a quantity of this alkaloid isolated as a by-product in the oxidation of commercial cinchonine (which contains hydrocinchonine) to the carboxylic acid cinchotenine.<sup>2</sup>

Considerable quantities of hydrocupreine were demanded in our synthetic work, and although this alkaloid is used in large amounts in

<sup>1</sup> Morgenroth and Levy, *Berl. klin. Wochschr.*, **48**, 1560, 1979 (1911).

<sup>2</sup> Skrap, *Ann.*, **197**, 376 (1879).

the preparation of its ethers<sup>1</sup> we were unable to find recorded any satisfactory method for obtaining this substance in fairly large quantities. Hesse<sup>2</sup> heated hydroquinine in sealed tubes with hydrochloric acid (sp. gr. 1.125) at 140–50° for 6–8 hours, while Pum<sup>3</sup> heated the dihydriodide with hydriodic acid (sp. gr. 1.7) on the water bath for 3 hours, but neither of these methods seemed suitable for the purpose in mind. Recourse was finally had to the well known method of de-etherifying by means of boiling aqueous hydrobromic acid, a process which could easily be applied to any quantity of hydroquinine. The details will be found in the experimental part.

As derivatives of the levorotatory alkaloid hydroquinine both hydrocupreine and its ethyl ether rotate the plane of polarized light to the left. It seemed, therefore, of interest to study the corresponding dextrorotatory stereoisomers, derivatives of hydroquinidine. We have been unable to find any description of these alkaloids, which we have named hydrocupreidine and ethylhydrocupreidine, although the former was evidently in the hands of Forst and Böhringer,<sup>4</sup> who heated hydroquinidine 6–8 hours at 140–50° with hydrochloric acid (sp. gr. 1.125) in a sealed tube and noted the evolution of methyl chloride and the presence in the tube of a mass of crystals from which a crystalline base could be isolated. No further description is given, however. We have found that hydrocupreidine is readily isolated by the method used for the preparation of its levorotatory isomer hydrocupreine, and that it may be converted without difficulty into its ethyl ether by means of diethyl sulfate and alcoholic alkali as described for the case of ethylhydrocupreine in German patent 254,712.

Another alkaloid selected for study was quinicine (quinotoxine). Quinidine was accordingly isomerized according to the specific instructions given by v. Miller, Rohde, and Fussenegger<sup>5</sup> for Pasteur's<sup>6</sup> original method. The first preparation was worked up in the usual

<sup>1</sup> *D. R. P.*, 254,712.

<sup>2</sup> *Ann.*, **241**, 279 (1887).

<sup>3</sup> *Monatsh.*, **16**, 73 (1895).

<sup>4</sup> *Ber.*, **15**, 1658 (1882).

<sup>5</sup> *Ibid.*, **33**, 3228 (1900).

<sup>6</sup> *Compt. rend.*, **37**, 110 (1853).

manner as the oxalate, but subsequently the quinine was isolated in good yield and without difficulty as the monohydrochloride, a salt which other workers have stated they were unable to obtain.

Finally, the preparation of isooctylhydrocupreine dihydrochloride was undertaken, this substance having been introduced in Germany during the war as "Vuzin"<sup>1</sup> and recommended for the treatment of infected wounds.

#### EXPERIMENTAL.

All of the values given for the optical rotation of the bases and salts were calculated by means of the formula  $[\alpha] = \alpha \times 100/l \times c$ , taking  $c$  as grams of substances per 100 cc. *solvent*, a close approximation for low concentrations.

*Cinchonine Hydrochloride*.—The base was suspended in hot water containing a few drops of alcohol and carefully neutralized to litmus on the water bath with hydrochloric acid. The salt separated from the filtered solution as spheres of woolly needles containing water of crystallization, part of which was slowly lost on air-drying. After coming to constant weight the crystals contained somewhat less than one molecule of water of crystallization. When rapidly heated to 215°, then slowly, the anhydrous salt softens at about 175° and melts at 217–8° with slow decomposition. In water  $[\alpha]_D^{20.5} = +177.4^\circ$ ,  $c = 1.083$ .

Subs., 0.6019: Loss 0.0224 at 100° *in vacuo* over H<sub>2</sub>SO<sub>4</sub>.

Calc. for C<sub>19</sub>H<sub>22</sub>ON<sub>2</sub>·HCl·H<sub>2</sub>O: H<sub>2</sub>O, 5.17. Found: 3.72.

Subs., (anhydrous), 0.2442: AgCl, 0.1064.

Calc. for C<sub>19</sub>H<sub>22</sub>ON<sub>2</sub>·HCl: Cl, 10.72. Found: 10.78.

*Cinchonidine Hydrochloride*.—The alkaloid was dissolved in boiling alcohol, neutralized to litmus with hydrochloric acid, and the solution concentrated to dryness *in vacuo*. The residue was taken up in hot absolute alcohol and treated with several volumes of dry ether, the salt separating on rubbing as radiating masses of needles which contained one molecule of water of crystallization when air-dry. When

<sup>1</sup> The original references, Morgenroth and Tugendreich, *Biochem. Z.*, **79**, 2577 (1917) and *Berl. klin. Wochschr.*, 1916, No. 29, are not available at the present writing.



rapidly heated to  $240^{\circ}$ , then slowly, the anhydrous compound softens at about  $160-70^{\circ}$  and melts with slow decomposition at  $242^{\circ}$ . In water  $[\alpha]_D^{20} = -117.6^{\circ}$ ,  $c = 1.214$ .

Subs., 0.7664: Loss 0.0400 at  $100^{\circ}$  *in vacuo* over  $H_2SO_4$ .

Calc. for  $C_{19}H_{22}ON_2 \cdot HCl \cdot H_2O$ :  $H_2O$ , 5.17. Found: 5.22.

Subs., (anhydrous), 0.2875:  $AgCl$ , 0.1233.

Calc. for  $C_{19}H_{22}ON_2 \cdot HCl$ :  $Cl$ , 10.72. Found: 10.61.

*Quinine Hydrochloride*.—Quinine was dissolved in alcohol, neutralized to litmus with hydrochloric acid, and the solution concentrated to dryness *in vacuo*. After recrystallization from water the salt showed the properties given by Hesse,<sup>1</sup> separating with 1.5 molecules of water of crystallization, melting to a jelly at  $154-60^{\circ}$  when anhydrous, and then also showing  $[\alpha]_D^{20} = -149.8^{\circ}$  in water,  $c = 1.322$ .

*Quinidine Hydrochloride*.—Our sample of the salt, prepared as in the case of the quinine hydrochloride, contained one molecule of water of crystallization as stated by Hesse.<sup>2</sup> The anhydrous salt gave  $[\alpha]_D^{20} = +200.8^{\circ}$  in water,  $c = 1.3$ , and when rapidly heated to  $255^{\circ}$ , then slowly, melted with decomposition at  $258-9^{\circ}$ , with preliminary darkening and sintering.

#### *Levorotatory Hydrogenated Cinchona Derivatives.*

*Hydroquinine*.—As stated in the introduction this substance was prepared according to the second method given in German patent 252,136. 40 g. of U. S. P. quinine were dissolved in 180 g. of 10% aqueous sulfuric acid and the solution was filtered and treated with 8–10 cc. of a 2% solution of palladious chloride, shaking until the precipitate first formed redissolved. The clear solution was then rinsed into a shaking apparatus, the air displaced by hydrogen, and the whole then shaken with free access of hydrogen under pressure of a column of water varying from about 25 to 60 cm. The reaction proceeded slowly until the palladium was entirely reduced, after which the absorption of hydrogen was quite rapid until almost the calculated amount had been absorbed. At the end the mixture was

<sup>1</sup> *Ann.*, 267, 143 (1892).

<sup>2</sup> *Ibid.*, 176, 225 (1875).

allowed to stand until the palladium had settled, after which the supernatant liquid was carefully decanted off and the palladium used again until its catalytic activity diminished. Ordinarily 8–10 cc. of the palladium chloride solution are sufficient for 5 or 6 reductions, after which the reduction proceeds so slowly that it is best to start with a new portion. The combined solutions from a number of reductions are filtered if necessary, diluted with a large volume of water, and rapidly made alkaline with sodium hydroxide. If the addition of alkali is too slow the neutral sulfate of hydroquinine is apt to crystallize out and contaminate the base. The amorphous precipitate of hydroquinine is filtered off on a large Büchner funnel, washed well with water, sucked as dry as possible, and then spread out to dry in the air. As so obtained the crude base contains about 5.5% of moisture, and, being stable to permanganate in sulfuric acid solution, is sufficiently pure for use in the preparation of hydrocupreine. The crude alkaloid may, if desired, be advantageously recrystallized from dry acetone, in which the amorphous substance is quite soluble and the crystalline form much less so. The dry, purified substance melts at  $171-2^{\circ}$ , as given in the literature for the natural alkaloid.

*Hydroquinine Hydrochloride*.—10 g. of commercial hydroquinine hydrochloride (purchased before the war) were dissolved in dry acetone, filtered from dirt, and treated with several volumes of dry ether. Crystallization was induced by rubbing, the salt separating as rhombic prisms. After air-drying the salt contained 0.5 molecule of water of crystallization. When rapidly heated to  $200^{\circ}$ , then slowly, the anhydrous salt melts with preliminary softening at  $206-8^{\circ}$  to a liquid filled with bubbles. It dissolves readily in water, alcohol, methyl alcohol, or acetone.  $[\alpha]_D^{21}$  of the dried salt is  $-123.9^{\circ}$  in water,  $c = 1.113$ . Hesse,<sup>1</sup> who prepared the salt from the sulfate and barium chloride describes it as long, flat prisms with two molecules of water of crystallization.

Subs., 0.7705: Loss, 0.0177, *in vacuo* at  $100^{\circ}$  over  $H_2SO_4$ .

Calc. for  $C_{20}H_{26}O_2N_2.HCl.1/2H_2O$ :  $H_2O$ , 2.42. Found: 2.30.

Subs., (anhydrous), 0.2630:  $AgCl$ , 0.1045.

Calc. for  $C_{20}H_{26}O_2N_2.HCl$ :  $Cl$ , 9.78. Found: 9.83.

<sup>1</sup> *Ann.*, 241, 261 (1887).

*Hydrocinchonidine Hydrochloride*.—The reduction of cinchonidine was carried out in the same way as that of hydroquinine, the yield being practically quantitative after recrystallization of the crude material from 50% alcohol. Ten g. of the base were suspended in hot water and neutralized with hydrochloric acid. The mixture was filtered, concentrated to dryness *in vacuo* and then redissolved in absolute alcohol and again concentrated as before, repeating this treatment in order to remove all of the water present. The residue was taken up in 50 cc. of absolute alcohol and cautiously treated with dry ether. The salt started to crystallize on rubbing and ligroin was then added in small amounts, with frequent rubbing, the salt gradually depositing as flat, microscopic needles and long, thin plates which were filtered off and washed with ligroin. The yield was 8 g. The salt contains no water, and when rapidly heated to 195°, then slowly, it melts at 202–3° with slight preliminary softening.  $[\alpha]_D^{26} = -89.4^\circ$  in water,  $c = 1.197$ . According to Hesse,<sup>1</sup> who probably obtained the salt by evaporation of an aqueous solution, it contains two molecules of water of crystallization, and, air-dry, shows  $[\alpha]_D^{20} = -80.4^\circ$ . In addition to the solubilities reported by Hesse it may be mentioned that the salt dissolves easily in dry chloroform and is somewhat less soluble in dry acetone.

Subs., 0.1541:  $\text{AgNO}_3$  soln. 9.13 cc. (1 cc. = 0.001812 g. Cl).

Calc. for  $\text{C}_{19}\text{H}_{24}\text{ON}_2\cdot\text{HCl}$ : Cl, 10.66. Found: 10.73.

*Hydrocupreine*.—As stated in the introduction the use of either hydrochloric or hydriodic acid for the demethylation of hydroquinine seemed scarcely suitable for preparative purposes, so the following method was adopted: 200 g. of crude hydroquinine were boiled 4 hours with 800 cc. of commercial aqueous hydrobromic acid (sp. gr. 1.49). If it is desired to collect the methyl bromide formed an air condenser may be used, connected with tubes leading into a freezing mixture. Otherwise it is advantageous to let the water liberated by the weakening of the acid boil off and attach the condenser only when the temperature of the boiling liquid has again reached 122–3°. The final dark brown solution is cooled and let stand overnight in

<sup>1</sup> *Ann.*, 214, 7, 15 (1882).



the ice box, filtering off the heavy, crystalline precipitate of hydrocupreine, dihydrobromide on cloth, washing with a little conc. hydrobromic acid, and distilling the filtrate down to about  $\frac{1}{3}$  of its original volume, a process requiring several hours and effecting the conversion of any unchanged hydroquinine present. The crop of crystals collected after cooling the concentrated liquid is filtered off and added to the first fraction, while the filtrate, in our experiments, was added to the hydrobromic acid used for the next preparation, instead of being worked up for the relatively small amount of hydrocupreine it contained. In this way the yield of subsequent preparations averages somewhat higher than that of the first. The combined fractions of the dihydrobromide are dissolved in about 8 liters of warm water, cooled, and cautiously treated with 10% sodium hydroxide solution until the localized precipitate first formed begins to dissolve slowly. Addition of sodium hydroxide is then continued rapidly, with vigorous stirring, until the copious precipitate of the base just redissolves in the excess of alkali. In this way the separation of a portion of the hydrocupreine as a gum is avoided, the gummy material dissolving in excess alkali only with the greatest difficulty. Boneblack is next added to collect a trace of gelatinous precipitate, and the solution is filtered, precipitating the hydrocupreine from the clear, yellow filtrate by the addition of an excess of saturated ammonium chloride solution. The amorphous base is filtered off on a large Büchner funnel, sucked as dry as possible, and added to about 750 cc. of boiling 95% alcohol. Most of the material dissolves before crystals begin to separate. At this point the solution is rapidly decanted from undissolved substance and this dissolved in the minimum amount of boiling alcohol and added to the rest. The mixture is then diluted with about 100 cc. of water, allowed to cool, and let stand overnight in the ice box, yielding 93 g. of crystalline hydrocupreine of a sufficiently high degree of purity for use in further work. On concentration of the mother liquors and washings to about  $\frac{1}{2}$  volume *in vacuo* and addition of about  $\frac{1}{4}$  volume of warm water a further crop of 21 g. was obtained, melting a few degrees below the main fraction. The ammoniacal filtrate from the crude base was shaken out with chloroform, the solvent removed, and the residue taken up in hot alcohol and cautiously diluted with water, yielding an additional 8.8 g.



of hydrocupreine. The total yield was thus 67% of the theory, calculating both the hydroquinine and hydrocupreine to the anhydrous basis. A portion of the main fraction, recrystallized from 85% alcohol, separated as compact aggregates of thick, minute, barely cream-colored plates which contain no water of crystallization. The substance swells and evolves gas at 185–90°, with preliminary softening, forming a glassy mass which adheres to the walls of the tube and only liquifies completely at 230°, with simultaneous darkening. A solution of the base in absolute alcohol is practically colorless and turns yellow when water is added. An aqueous suspension gives a rather weak brown color with ferric chloride.  $[\alpha]_D^{23}$  in absolute alcohol is  $-148.7^\circ$ ,  $c = 1.13$ .

Subs., 0.1708: N, 13.6 cc. (24.0°, 761 mm.).

Calc. for  $C_{19}H_{24}O_2N_2$ : N, 8.97. Found: N, 9.17.

According to Hesse<sup>1</sup> hydrocupreine separates with two molecules of water of crystallization, melts at 168–70°, and gives a dark brown color with ferric chloride. Pum<sup>1</sup> gives the melting point as 170°, while Giemsa and Halberkann,<sup>2</sup> who obtained the base by the catalytic reduction of cupreine, describe it as forming needles melting at 204°, with  $[\alpha]_D^{20} = -154.8^\circ$  in absolute alcohol,  $c = 1.044$ .

*Hydrocupreine Hydrochloride*,  $C_{19}H_{24}O_2N_2 \cdot HCl$ .—As far as we have been able to find, this salt has never been described. Hydrocupreine was suspended in hot water and neutralized at the boiling point with 10% hydrochloric acid. The resulting mixture was filtered, cooled, and the precipitate recrystallized from boiling water containing a few drops of dilute hydrochloric acid to drive back the slight dissociation which otherwise occurred. The salt separated slowly as radiating masses of needles which did not contain water of crystallization. When rapidly heated the compound blackens above 255° and melts and decomposes at about 280°. In absolute alcohol  $[\alpha]_D^{22.5} = -132.3^\circ$ ,  $c = 0.945$ . The salt is difficultly soluble in cold water, acetone, or chloroform, but dissolves more readily in alcohol and still more easily in methyl alcohol. The aqueous solution is yellow, as was found by Hesse<sup>1</sup> for the other neutral salts.

<sup>1</sup> *Loc. cit.*

<sup>2</sup> *Ber.*, 51, 1329 (1918).

Subs., 0.1515:  $\text{AgNO}_3$  soln. 8.49 cc. (1 cc. = 0.001812 g. Cl).

Calc. for  $\text{C}_{19}\text{H}_{24}\text{O}_2\text{N}_2\text{HCl}$ : Cl, 10.17. Found: 10.15.

*Hydrocupreine Dihydrobromide*.—A portion of the first crop of crude hydrocupreine dihydrobromide obtained in the preparation of the alkaloid (see above) was dissolved in boiling water containing a little hydrobromic acid, boneblackd, and filtered. From the faintly yellow, supersaturated solution obtained on cooling the salt separates after seeding as leaf-like aggregates of irregular prisms which contain two molecules of water of crystallization when air-dry. The anhydrous salt turns yellow and softens to a jelly at about  $180\text{--}90^\circ$ , gradually becoming fluid as the temperature is further raised.

Subs., (air-dry), 0.7565: Loss, 0.0533, *in vacuo* at  $100^\circ$  over  $\text{H}_2\text{SO}_4$ .

Calc. for  $\text{C}_{19}\text{H}_{24}\text{O}_2\text{N}_2 \cdot 2\text{HBr} \cdot 2\text{H}_2\text{O}$ :  $\text{H}_2\text{O}$ , 7.06. Found: 7.05.

Subs., (anhydrous), 0.1144:  $\text{AgBr}$ , 0.0904.

Calc. for  $\text{C}_{19}\text{H}_{24}\text{O}_2\text{N}_2 \cdot 2\text{HBr}$ : Br, 33.73. Found: 33.62.

The air-dry salt is fairly easily soluble in water, while the anhydrous substance dissolves readily in absolute alcohol or methyl alcohol and is practically insoluble in chloroform or dry acetone.

*Hydrocupreine Nitrate*,  $\text{C}_{19}\text{H}_{24}\text{O}_2\text{N}_2 \cdot \text{HNO}_3$ .—Five g. of hydrocupreine were suspended in a little dil. alcohol and neutralized to litmus with dil. nitric acid, finally warming on the water bath to dissolve all of the base. The solution was concentrated to dryness *in vacuo* and the residue dried in a vacuum desiccator. After dissolving in warm, dry acetone and filtering, the solution was seeded with crystals obtained by letting some of the crude product stand overnight in dry acetone. The salt was deposited slowly as a hard crust of rosetts of flat needles which were anhydrous when air-dried. The yield was 3.2 g. When rapidly heated to  $215^\circ$ , then slowly, the compound melts to a dark liquid at  $220\text{--}2^\circ$ . It turns pink on exposure to direct sunlight, is quite freely soluble in water, and dissolves readily in alcohol or methyl alcohol, less easily in dry acetone, and very sparingly in dry chloroform or benzene.

Subs., 0.1195: N, 11.6 cc. ( $22.0^\circ$ , 754 mm.).

Calc. for  $\text{C}_{19}\text{H}_{24}\text{O}_2\text{N}_2 \cdot \text{HNO}_3$ : N, 11.20. Found: 11.15.

*Ethylhydrocupreine Hydrochloride*,  $\text{C}_{21}\text{H}_{28}\text{O}_2\text{N}_2 \cdot \text{HCl}$ .—The ethylhydrocupreine and amorphous ethylhydrocupreine hydrochloride used

in the preparation of the 4 derivatives of "optochin" here recorded were purchased in the open market before the war. As stated by Giemsa and Halberkann<sup>1</sup> the hydrochloride on the market is amorphous, but these authors found that if dissolved in water or alcohol and evaporated it was left behind as radiating needles. We have found it a simple matter to obtain the pure, crystalline salt as follows: The commercial hydrochloride is dissolved in boiling, dry acetone, filtered from dirt, and the solution treated with several volumes of dry ether. The salt separates on scratching as rhombic crystals which are anhydrous when air-dried. It melts at 252–4° to a brown, turbid liquid which rapidly clears, and in water shows  $[\alpha]_D^{21} = -123.6^\circ$ ,  $c = 0.959$ . The salt dissolves readily in water, alcohol, or methyl alcohol, less easily in dry acetone.

Subs., 0.2434: AgCl, 0.0935.

Calc. for  $C_{21}H_{28}O_2N_2 \cdot HCl$ : Cl, 9.41. Found: 9.50.

*Ethylhydrocupreine Hydrobromide*.—The commercial hydrochloride was dissolved in a little hot water and treated with conc. potassium bromide solution until a faint turbidity just persisted. On cooling and letting stand part of the hydrobromide separated as an oil which gradually crystallized, the process being accelerated by rubbing. The crystals were filtered off, washed with a little ice-cold water, and recrystallized from water, separating slowly on seeding and letting stand at 0° as aggregates of rhombs which contain no water of crystallization. When rapidly heated to 255°, then slowly, the salt melts to a dark liquid at 258–9°, with slight preliminary darkening and sintering. It dissolves sparingly in cold water, readily on boiling, and is also easily soluble in methyl or ethyl alcohol or chloroform.

Subs., 0.1196: AgNO<sub>3</sub> soln. 5.65 cc. (1 cc. = 0.004043 g. Br).

Calc. for  $C_{21}H_{28}O_2N_2 \cdot HBr$ : Br, 18.96. Found: 19.11.

*Ethylhydrocupreine Dihydrobromide*.—One g. of ethylhydrocupreine was dissolved in a little water with the aid of enough conc. hydrobromic acid to give a strong blue color with congo-red. The solution was boneblackened to remove a slight turbidity and set in the

<sup>1</sup> *Loc cit.*



ice box, with occasional rubbing. The salt separated slowly as pale greenish yellow crusts of rhombic crystals containing 0.5 molecule of water of crystallization.

Subs., 0.8541: Loss, 0.0157, *in vacuo* at room temp. over  $\text{H}_2\text{SO}_4 + \text{NaOH}$ .

Calc. for  $\text{C}_{21}\text{H}_{28}\text{O}_2\text{N}_2 \cdot 2\text{HBr} \cdot \frac{1}{2}\text{H}_2\text{O}$ :  $\text{H}_2\text{O}$ , 1.76. Found: 1.84.

Subs., (anhydrous), 0.1221:  $\text{AgBr}$ , 0.0905.

Calc. for  $\text{C}_{21}\text{H}_{28}\text{O}_2\text{N}_2 \cdot 2\text{HBr}$ :  $\text{Br}$ , 31.85. Found: 31.55.

*Ethylhydrocupreine Methiodide*,  $\text{C}_{21}\text{H}_{28}\text{O}_2\text{N}_2 \cdot \text{CH}_3\text{I}$ .—17 g. of ethylhydrocupreine were dissolved in acetone and treated with 7.2 g. of methyl iodide. Heat was evolved and the quaternary salt separated almost immediately as glistening crystals. After standing overnight the methiodide was filtered off and washed with acetone. The yield was 21.5 g. A portion was recrystallized from absolute alcohol, adding a little dry ether to the warm solution before crystallization commenced. The methiodide forms glistening platelets with a faint yellow tinge and melts at  $195-6^\circ$  to a yellow-brown liquid.  $[\alpha]_D^{21.5}$  in absolute alcohol is  $-113.0^\circ$ ,  $c = 0.992$ . The compound dissolves somewhat sparingly in water, more easily in absolute alcohol, and very readily in methyl alcohol or chloroform.

Subs., 0.2465:  $\text{AgI}$ , 0.1200.

Calc. for  $\text{C}_{22}\text{H}_{31}\text{O}_2\text{N}_2\text{I}$ :  $\text{I}$ , 26.32. Found: 26.31.

*Sec.-Octylhydrocupreine Dihydrochloride* ("Vuzin").—10.4 g. of hydrocupreine were suspended in 35 cc. of absolute alcohol and treated with 3.8 g. of 50% potassium hydroxide solution. The mixture was warmed until clear, cooled in ice-water, and then treated with 8 g. of secondary octyl iodide,  $\text{C}_6\text{H}_{13}(\text{CH}_2)\text{CHI}$ , the general procedure being one of the alternatives outlined in German patent 254,712. The solution was allowed to stand at the temperature of a warm room for a month and was then chilled to  $0^\circ$  and filtered, 3.3 g. of potassium iodide remaining on the filter. The filtrate was diluted with an excess of dil. hydrochloric acid and shaken out with ether to remove isooctyl iodide and isooctyl alcohol, after which the aqueous liquid was made strongly alkaline with sodium hydroxide and again shaken out with ether. The ethereal extract was allowed to stand for several days, being then poured from a few drops of a dark,



aqueous solution that had separated and extracted with 5% hydrochloric acid. The acid extract was partially concentrated *in vacuo* but the concentration could not be carried far owing to gelatinization of the solution. On standing overnight the dihydrochloride began to crystallize, and the transformation from the gelatinous into the crystalline form was completed by letting stand for several days in a warm place and finally cooling to room temperature. The salt was filtered off, washed with a little very dil. hydrochloric acid, and recrystallized from water, adding a few drops of conc. hydrochloric acid after cooling. The salt forms faintly yellow sheaves and rosets of delicate needles which contain two molecules of water of crystallization when air-dry. The yield was 6 g. The anhydrous salt softens slightly above 140°, melts to a pale yellow jelly at 157–60°, and liquefies completely at 190–5°, with slight gas evolution. It dissolves readily in cold absolute alcohol, methyl alcohol, or dry chloroform, sparingly in cold dry acetone, more easily on boiling, and gelatinizes with a little benzene. The air-dry salt dissolves rather sparingly in cold water, readily on warming. The solution has a salty taste and gives precipitates with potassium dichromate and picric acid. A clear, warm, dilute aqueous solution soon becomes turbid, presumably owing to the separation of the monohydrochloride, as it clears again on heating. A dilute solution, treated with a few drops of aqueous hydrobromic acid, deposits the dihydrobromide on rubbing as spherical aggregates of microscopic needles.

Subs., (air-dry), 0.7612: Loss, 0.0467, *in vacuo* at room temp. over  $\text{H}_2\text{SO}_4$ .

Calc. for  $\text{C}_{27}\text{H}_{40}\text{O}_2\text{N}_2 \cdot 2\text{HCl} \cdot 2\text{H}_2\text{O}$ :  $\text{H}_2\text{O}$ , 6.76. Found: 6.14.

Subs., (anhydrous), 0.1421: N, 7.1 cc. (21.0°, 750 mm.).

Subs., 0.1359:  $\text{AgNO}_3$  soln. 10.9 cc. (1 cc. = 0.001794 g. Cl).

Calc. for  $\text{C}_{27}\text{H}_{40}\text{O}_2\text{N}_2 \cdot 2\text{HCl}$ : N, 5.63; Cl, 14.26. Found: N, 5.73; Cl, 14.38.

#### *Dextrorotatory Hydrogenated Cinchona Derivatives.*

*Hydrocinchonine Hydrochloride*.—Two samples of this salt were prepared by neutralizing the base with hydrochloric acid in boiling water suspension; one from the naturally occurring alkaloid recovered as a by-product in the oxidation of commercial cinchonine to cinchotenine,<sup>1</sup> and the other from hydrocinchonine prepared from cin-

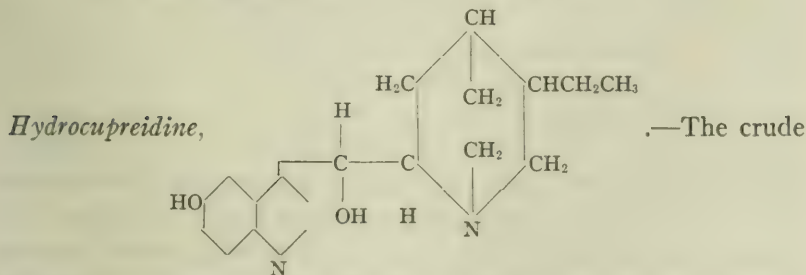
<sup>1</sup> *Loc. cit.*

choning by reduction with palladium and hydrogen as in the case of hydroquinine. Both samples gave good analyses, and conformed to the description given by Forst and Böhringer,<sup>1</sup> forming delicate needles containing two molecules of water of crystallization. The anhydrous hydrochloride prepared from the natural alkaloid darkened above 180°, melted at 220–1° to a dark liquid, and showed  $[\alpha]_D^{23} = +155.2^\circ$  in water,  $c = 0.796$ , while the anhydrous salt from the synthetic base darkened slightly above 200°, melted at 221–3° to a dark liquid, with slight gas evolution, and showed  $[\alpha]_D^{25} = +159.3^\circ$  in water,  $c = 0.741$ . A mixed melting-point determination showed no material depression, so it appears certain that the two preparations represent one and the same substance, as was to be expected.<sup>2</sup> v. Arlt<sup>3</sup> gives the melting point of hydrocinchonine hydrochloride as 216°.

*Hydroquinidine Hydrochloride.*—The salt was prepared from catalytically reduced quinidine, neutralized with hydrochloric acid in 50% alcoholic suspension, concentrated to dryness *in vacuo*, and recrystallized from water, separating as rhombic crystals which contained no water of crystallization. The salt was first described by Forst and Böhringer,<sup>4</sup> to whose description the following data are added: It darkens at about 270° and decomposes at 273–4°.  $[\alpha]_D^{26}$  in water is  $+183.9^\circ$ ,  $c = 1.278$ . The salt dissolves easily in cold methyl alcohol or chloroform, less readily in water or absolute alcohol, and difficultly in dry acetone.

Subs., 0.1538:  $\text{AgNO}_3$  soln. 8.40 cc. (1 cc. = 0.001812 g. Cl).

Calc. for  $\text{C}_{20}\text{H}_{26}\text{O}_2\text{N}_2\cdot\text{HCl}$ : Cl, 9.78. Found: 9.90.



<sup>1</sup> *Ber.*, 14, 437 (1881).

<sup>2</sup> *D. R. P.*, 234,137.

<sup>3</sup> *Monatsh.*, 20, 431 (1899).

<sup>4</sup> *Ber.*, 15, 1657 (1882).

hydroquinidine used for the preparation of this alkaloid was obtained in precisely the same way as the hydroquinine used in the preparation of hydrocupreine, the levorotatory stereoisomer of hydrocupreidine. 77 g. of the crude hydroquinidine were dissolved in 320 cc. of commercial 40% hydrobromic acid. The solution was boiled until the temperature of the liquid had reached 125° before attaching an air-condenser to the flask. After another three-quarters of an hour a heavy precipitate of hydrocupreidine dihydrobromide suddenly separated. After cooling the product was filtered off, washed with several small portions of 40% hydrobromic acid, and the filtrate and washings distilled until a second crop separated. This was filtered off and washed as in the case of the first crop, and the filtrate again concentrated to about  $\frac{1}{2}$  volume, when a further amount of the salt was obtained on cooling and seeding. The combined fractions of the dihydrobromide were then dissolved in 3-4 liters of water, made alkaline with an excess of sodium hydroxide, and the base precipitated with ammonium chloride solution, using the same precautions as in the case of hydrocupreine. The crude base began to crystallize spontaneously on letting stand overnight and was recrystallized by dissolving in hot 95% alcohol and adding water until the initial turbidity just redissolved. In this way a first crop of 36.5 g. was obtained, while dilution of the mother liquors yielded further amounts which, when recrystallized as above, totaled 15.3 g., making the entire yield of purified base 51.8 g. For characterization a portion of the substance was converted into the hydrochloride (see below) and a part of this dissolved in warm water, precipitated with a slight excess of aqueous ammonia, and recrystallized from 50% alcohol, forming glistening, cream-colored hexagonal plates which were air-dried and then contained between 0.5 and 1.0 molecule of water of crystallization. The anhydrous alkaloid does not melt sharply, softening to a jelly above 170°, and becoming completely fluid at about 195°. It is readily soluble in cold absolute alcohol or methyl alcohol, sparingly in the cold in dry acetone or chloroform, more easily on heating, and is quite difficultly soluble in dry ether.  $[\alpha]_D^{19.5}$  of the anhydrous base in absolute alcohol is + 253.4°,  $c = 1.422$ . In all its chemical properties the alkaloid shows a strict analogy to its levorotatory stereoisomer, hydrocupreine. Like this, its absolute



alcoholic solution is practically colorless and turns yellow on adding water. An aqueous suspension also gives a rather weak brown color with ferric chloride.

Subs., (air-dry), 0.1052: Loss, 0.0048, *in vacuo* at 110° over  $\text{H}_2\text{SO}_4$ .

Calc. for  $\text{C}_{19}\text{H}_{24}\text{O}_2\text{N}_2 \cdot \text{H}_2\text{O}$ :  $\text{H}_2\text{O}$ , 5.45. Found: 4.56.

Subs., (anhydrous), 0.1004:  $\text{CO}_2$ , 0.2700;  $\text{H}_2\text{O}$ , 0.0678.

Subs., (anhydrous), 0.1353: N, 10.7 cc. (20.0°, 741 mm.).

Calc. for  $\text{C}_{19}\text{H}_{24}\text{O}_2\text{N}_2$ : C, 73.03; H, 7.75; N, 8.97. Found: C, 73.34; H, 7.56; N, 8.99.

*Hydrocupreidine Hydrochloride*,  $\text{C}_{19}\text{H}_{24}\text{O}_2\text{N}_2 \cdot \text{HCl} \cdot \text{H}_2\text{O}$ .—9.5 g. of crystalline hydrocupreidine were suspended in about 50 cc. of 50% alcohol and neutralized with dil. hydrochloric acid, finally warming on the water bath. The hydrochloride, which separated on cooling, was filtered off and recrystallized from 50% alcohol, separating slowly as rosets and sheaves of prismatic needles containing one molecule of water of crystallization. The yield was 7.7 g. When rapidly heated to 230°, then slowly, the anhydrous salt melts at 231–3°, with darkening.  $[\alpha]_D^{24}$  in water is +194.2°,  $c = 0.618$ . It dissolves rather sparingly in dry acetone or chloroform and in cold absolute alcohol or water, but dissolves in the last solvents on warming. The aqueous solution is yellow, like the solutions of the neutral salts of hydrocupreine. 1.8 g. were recovered from the mother liquors of the recrystallization by adding saturated sodium chloride solution.

Subs., (air-dry), 0.6133: Loss, 0.0291, *in vacuo* at 100° over  $\text{H}_2\text{SO}_4$ .

Calc. for  $\text{C}_{19}\text{H}_{24}\text{O}_2\text{N}_2 \cdot \text{HCl} \cdot \text{H}_2\text{O}$ :  $\text{H}_2\text{O}$ , 4.91. Found: 4.75.

Subs., (anhydrous), 0.2109: AgCl, 0.0855.

Calc. for  $\text{C}_{19}\text{H}_{24}\text{O}_2\text{N}_2 \cdot \text{HCl}$ : Cl, 10.17. Found: 10.03.

*Hydrocupreidine Dihydrobromide*.—A portion of the first fraction of this salt obtained in the preparation of hydrocupreidine (see above) was recrystallized from water, adding about  $\frac{1}{2}$  volume of 40% hydrobromic acid to the cooled, filtered solution. The dihydrobromide separated after seeding as faintly yellow, glistening plates which contained no water of crystallization. When heated the salt turns yellow, but does not melt below 275°. It is quite freely soluble in cold water, rather difficultly in boiling absolute alcohol, and apparently insoluble in dry acetone or chloroform.



Subs., 0.1302: AgBr, 0.1030.

Calc. for  $C_{19}H_{24}O_2N_2 \cdot 2HBr$ : Br, 33.73. Found: 33.67.

*Hydrocupreidine Hydriodide*.—1.8 g. of hydrocupreidine hydrochloride were dissolved in hot water and treated with several grams of sodium iodide. The hydriodide separated on cooling and scratching and was recrystallized from water, forming faintly pinkish rhombic plates and prisms containing one molecule of water of crystallization. The anhydrous salt melts at  $209-12^\circ$  to a brown liquid which slowly decomposes. It dissolves sparingly in boiling water with a yellow color, more easily in boiling absolute alcohol, separating on cooling as rhombic prisms, and also dissolves in the cold in dry acetone with a bright yellow color.

Subs., (air-dry), 0.9327: Loss, 0.0367, *in vacuo* at  $100^\circ$  over  $H_2SO_4$ .

Calc. for  $C_{19}H_{24}O_2N_2 \cdot HI \cdot H_2O$ :  $H_2O$ , 3.93. Found: 3.93.

Subs., (anhydrous), 0.1823: AgI, 0.0961.

Calc. for  $C_{19}H_{24}O_2N_2 \cdot HI$ : I, 28.84. Found: 28.49.

*Hydrocupreidine Nitrate*.—The base was dissolved in a small volume of 95% alcohol, cooled, diluted with a little water, and neutralized to litmus with 10% aqueous nitric acid, adding a little water from time to time until the final strength of the alcohol present was about 33%. The salt crystallized on rubbing and was filtered off after standing in the ice box, washed with a little 33% alcohol, and recrystallized from 50% alcohol, separating slowly on cooling as cream-colored rhombs containing one molecule of water of crystallization. The air-dry salt dissolves in boiling water with a yellow color but is only sparingly soluble in the cold, while the anhydrous nitrate is readily soluble in absolute alcohol or methyl alcohol and very sparingly so in dry chloroform, acetone, or benzene. When rapidly heated to  $150^\circ$ , then slowly, it turns yellow and gradually softens, forming a jelly at about  $160^\circ$  and liquefying completely at  $175-80^\circ$ .

Subs., (air-dry), 0.6215: Loss, 0.0287, *in vacuo* at  $80^\circ$  over  $H_2SO_4$ .

Calc. for  $C_{19}H_{24}O_2N_2 \cdot HNO_3 \cdot H_2O$ :  $H_2O$ , 4.58. Found: 4.62.

Subs., (anhydrous), 0.1271: N, 12.2 cc. ( $23.0^\circ$ , 758 mm.).

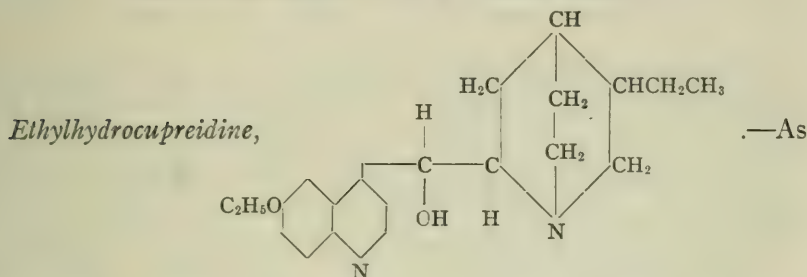
Calc. for  $C_{19}H_{24}O_2N_2 \cdot HNO_3$ : N, 11.20. Found: 11.03.

*Hydrocupreidine Methiodide*,  $C_{19}H_{24}O_2N_2 \cdot CH_3I$ .—1.6 g. of crystalline hydrocupreidine were dissolved in warm absolute alcohol, cooled, and

treated with 0.8 g. of methyl iodide. The solution was allowed to stand for 5 days in a dark place, after which the deposit of 2.0 g. of yellow, rhombic crystals of the methiodide was filtered off and washed with a little absolute alcohol. A portion was pulverized and dissolved in boiling water, in which it is difficultly soluble, separating on cooling as glistening prisms which darken slightly when rapidly heated and melt and decompose at about  $295^{\circ}$  only when left in the hot bath for a few moments. The methiodide is more soluble in 50% alcohol than in water or absolute alcohol, and also dissolves in methyl alcohol.  $[\alpha]_D^{20}$  in 50% alcohol is  $+202.6$ ,  $c = 0.555$ .

Subs., 0.2009; AgI, 0.1034.

Calc. for  $C_{20}H_{27}O_2N_2I$ : I, 27.94. Found: 27.82.



stated in the introduction, hydrocupreidine was converted into its ethyl ether by one of the alternative methods given in German patent 254,712. 32 g. of air-dry hydrocupreidine (+ 1 H<sub>2</sub>O) were suspended in 90 cc. of absolute alcohol and treated with 11.5 g. of 50% aqueous potassium hydroxide solution. After stirring until clear and chilling in ice-water 13.5 cc. of washed diethyl sulfate were added and the solution placed in the ice box. The gelatinous mass first formed gradually changed over into crystals of the ethylhydrocupreidine. After 9 days the new alkaloid was filtered off, washed with a little cold alcohol, let stand for several hours with 50% alcohol containing sodium hydroxide solution, and finally washed with 50% alcohol and water. The yield was 12.7 g. The combined filtrates were made strongly alkaline and shaken out with ether, yielding only an additional trace of ethylhydrocupreidine, but on adding saturated ammonium chloride solution to the aqueous layer and shaking out the precipitated gum with ether 3 g. of hydrocupreidine were recovered.

Deducting for water and recovered hydrocupreidine, the yield of the ethyl ether is 42.3% of the theory. A portion of the base was recrystallized twice from 50% alcohol and then from benzene, separating as rosetts and sheaves of delicate needles which melt constantly at 197.5–8.0° with slight preliminary softening, resolidifying again a few degrees below the melting point. It dissolves quite freely in cold chloroform or methyl alcohol, rather sparingly in the cold in absolute alcohol, benzene, or ethyl acetate, easily on boiling, and is less soluble in dry acetone.  $[\alpha]_D^{23.5}$  in absolute alcohol is + 212.8,  $c = 1.008$ .

Subs., 0.1151: CO<sub>2</sub>, 0.3147; H<sub>2</sub>O, 0.0849.

Subs., 0.1399: N, 10.2 cc. (23.0°, 763 mm.).

Calc. for C<sub>21</sub>H<sub>23</sub>O<sub>2</sub>N<sub>2</sub>: C, 74.07; H, 8.29; N, 8.24. Found: C, 74.57; H, 8.25; N, 8.45.

*Ethylhydrocupreidine Hydrochloride*.—Four g. of crystalline ethylhydrocupreidine were suspended in boiling water, treated with dil. hydrochloric acid until neutral to litmus, filtered from a small amount of insoluble material, and rapidly cooled. The hydrochloride which separated was collected and recrystallized from a small volume of 50% alcohol, separating after seeding and standing in the ice box as nacreous aggregates of flat needles and long, narrow plates. The yield was 3.5 g. The air-dry salt contains 4 molecules of water of crystallization and has a not very pronounced, bitter taste. After drying  $[\alpha]_D^{22}$  is + 183.3° in water,  $c = 0.592$ , and when rapidly heated it sinters to a jelly at 140–55° and melts at 258–60° to a red-brown liquid. The dried salt dissolves very easily in absolute alcohol, methyl alcohol, or dry chloroform, difficultly in dry acetone, and sparingly in cold benzene, but quite easily on warming. It also dissolves fairly readily in cold water, the hydrate soon separating. A dilute aqueous solution gives precipitates with picric acid and potassium dichromate. When the salt is recrystallized from water it separates slowly as rhombic crystals.

Subs., (air-dry), 0.7177: Loss, 0.1150, *in vacuo* at 100° over H<sub>2</sub>SO<sub>4</sub>.

Calc. for C<sub>21</sub>H<sub>23</sub>O<sub>2</sub>N<sub>2</sub>.HCl.4H<sub>2</sub>O: H<sub>2</sub>O, 16.05. Found: 16.03.

Subs., (anhydrous), 0.1387: N, 9.1 cc. (23.0°, 762 mm.).

Subs., (anhydrous), 0.1242: AgNO<sub>3</sub> soln. 6.53 cc. (1 cc. = 0.001794 g. Cl).

Calc. for C<sub>21</sub>H<sub>23</sub>O<sub>2</sub>N<sub>2</sub>.HCl: N, 7.44; Cl, 9.41. Found: N, 7.59; Cl, 9.43.



*Ethylhydrocupreidine Hydrobromide*.—0.7 g. of the hydrochloride was dissolved in hot water and treated with about 2 g. of potassium bromide. 0.5 g. of the hydrobromide separated from the still hot solution on rubbing as rhombic crystals containing no water of crystallization. When rapidly heated to  $248^{\circ}$ , then slowly, the salt melts at  $250.5-3^{\circ}$  with slow decomposition. It dissolves readily in cold, dry chloroform or methyl alcohol, less easily in absolute alcohol, and very sparingly in cold water, more readily on boiling.

Subs., 0.1198:  $\text{AgNO}_3$  soln. 5.70 cc. (1 cc. = 0.004043 g. Br).

Calc. for  $\text{C}_{21}\text{H}_{28}\text{O}_2\text{N}_2\cdot\text{HBr}$ : Br, 18.96. Found: 19.23.

*Ethylhydrocupreidine Dihydrobromide*.—1.5 g. of crystalline base were dissolved in water with the aid of an excess of hydrobromic acid. Crystallization soon started, and the collected salt was recrystallized from water, adding about  $\frac{1}{3}$  volume of 40% hydrobromic acid after cooling. When seeded the solution rapidly deposited the dihydrobromide as radiating masses of delicate, silky needles containing 0.5 molecule of water of crystallization. The yield was 1.3 g. The dried salt gradually turns yellow above  $130^{\circ}$ , melts to a jelly at about  $175-85^{\circ}$ , and swells and evolves gas at  $200-5^{\circ}$ . It dissolves readily in water, alcohol, or methyl alcohol, is somewhat soluble in boiling dry chloroform, and is practically insoluble in dry acetone.

Subs., 1.4083: Loss, 0.0292, *in vacuo* at room temp. over  $\text{H}_2\text{SO}_4$  and NaOH.

Calc. for  $\text{C}_{21}\text{H}_{28}\text{O}_2\text{N}_2\cdot 2\text{HBr}\cdot\frac{1}{2}\text{H}_2\text{O}$ :  $\text{H}_2\text{O}$ , 1.76. Found: 2.07.

Subs., (anhydrous), 0.1246:  $\text{AgBr}$ , 0.0921.

Calc. for  $\text{C}_{21}\text{H}_{28}\text{O}_2\text{N}_2\cdot 2\text{HBr}$ : Br, 31.85. Found: 31.45.

*Ethylhydrocupreidine Methiodide*.—0.6 g. of the crystalline alkaloid was dissolved in warm alcohol, cooled, and treated with 0.3 g. of methyl iodide. After letting stand overnight the solution was diluted with water, but as no precipitation occurred the solution was concentrated on the water bath until most of the alcohol had escaped. The crystals obtained on cooling were filtered off, washed with a little water, and recrystallized, with boneblackening, from 50% alcohol, separating as rhombs and prisms showing  $[\alpha]_{\text{D}}^{22} = +189.6^{\circ}$  in methyl alcohol,  $c = 1.131$ . When rapidly heated to  $252^{\circ}$ , then



slowly, the methiodide decomposes at  $253-5^{\circ}$ . It dissolves rather sparingly in boiling water, more easily in boiling alcohol or cold methyl alcohol, and is practically insoluble in boiling dry acetone.

Subs., 0.1365:  $\text{AgNO}_3$  soln. 5.6 cc. (1 cc. = 0.006421 g. I).

Calc. for  $\text{C}_{22}\text{H}_{31}\text{O}_2\text{N}_2\text{I}$ : I, 26.32. Found: 26.34.

*Quinicine Hydrochloride*,  $\text{C}_{20}\text{H}_{24}\text{O}_2\text{N}_2\cdot\text{HCl}$ .—This salt has apparently never been prepared, but as will be set forth below, it may be isolated so easily and in such good yield that the customary isolation of quinicine as the sparingly soluble oxalate would seem unnecessary, especially as the oxalic acid present must often be removed before the alkaloid can be used in subsequent operations. Our first sample of the hydrochloride was prepared from quinicine oxalate<sup>1</sup> as follows: The salt was dissolved in hot water, treated with a slight excess of calcium chloride, and boneblackened and filtered. After concentrating to dryness *in vacuo* the residue was taken up in absolute alcohol and again concentrated as before. The deposit of crystals was taken up in hot absolute alcohol, filtered from a trace of precipitate which was collected with the aid of boneblack, and dry ether added to the filtrate until the initial turbidity dissolved with difficulty. The salt separated on seeding and rubbing as arborescent aggregates of minute leaflets which melted at  $179-80^{\circ}$  and showed  $[\alpha]_D^{20} = +16.26^{\circ}$  in water,  $c = 0.80$ . A subsequent preparation was obtained directly as follows:

112 g. of quinidine (100 g., calculated to the anhydrous basis) were converted into quinicine according to the method of v. Miller, Rohde, and Fussenegger,<sup>1</sup> and the crude, viscous base taken up without further treatment in about 3 volumes of absolute alcohol and neutralized with absolute alcoholic hydrochloric acid until a test drop proved neutral on wet litmus paper. After seeing with a few crystals of the preparation obtained through the oxalate the hydrochloride separated rapidly on rubbing and letting stand in the ice box. 74 g. were obtained after washing with a little absolute alcohol. A portion, recrystallized from absolute alcohol, separated in the form given above and did not contain water of crystallization. It melted with

<sup>1</sup> *Loc. cit.*

decomposition at  $180-2^{\circ}$  and showed  $[\alpha]_D^{20} = +13.7^{\circ}$  in water,  $c = 1.861$ . It dissolves readily in water or methyl alcohol, less easily in absolute alcohol, and is practically insoluble in dry acetone.

Subs., 0.1395: N, 9.4 cc. ( $21.5^{\circ}$ , 770 mm.).

Subs., 0.1817: AgCl, 0.0717.

Calc. for  $C_{20}H_{24}O_2N_2 \cdot HCl$ : N, 7.77; Cl, 9.83. Found: N, 7.91; Cl, 9.76.



## PREPARATION OF RHAMNOSE.

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Methods for the preparation of rhamnose were published by Kruis<sup>1</sup> and by Rayman.<sup>2</sup> These authors found that commercial extract of black oak bark known as "quercitron extract" was a convenient material for the preparation of this sugar. The process employed by these authors was as follows.

Quercitron extract was hydrolyzed by boiling with dilute sulfuric acid for  $\frac{1}{2}$  hour. This solution was filtered and neutralized with calcium or barium carbonate. The solution after filtering was concentrated to a thick syrup and allowed to crystallize.

When this process was applied to material obtainable at the present time the result was very unsatisfactory. However, it was found possible to obtain quite satisfactory yields of rhamnose by a modification of the original method. The modified process is as follows.

2 kilos of the liquid quercitron extract,<sup>3</sup> which has the consistency of a thick syrup, is dissolved in enough hot water to make  $5\frac{1}{2}$  liters. It is brought to a boil and 180 cc. of concentrated sulfuric acid dissolved in 320 cc. of water are gradually added, thus making the concentration of the acid 3 per cent by volume. The mixture is gently boiled for 30 minutes, then poured into a crock jar or enameled kettle, and quickly cooled.

The liquid after being separated from the insoluble material is neutralized with barium hydroxide. The barium sulfate is filtered off, the filtrate evaporated to a thin syrup (approximately 350 cc.), and 8 volumes of 95 per cent alcohol are slowly added with constant

<sup>1</sup> Kruis, K., *Sitzungsberichte der Böhmisches Gesellschaft der Wissenschaften*, Prague, 1877-78, 157.

<sup>2</sup> Rayman, B., *Bull. Soc. Chim.*, 1887, xlvii, 668.

<sup>3</sup> The material used in this work was obtained from A. Klipstein and Co. of New York.



stirring. The heavy precipitate formed is filtered off and sucked dry on a Büchner funnel. The resulting filtrate is evaporated under diminished pressure to a thick syrup, dissolved in 1 liter of 95 per cent alcohol, and to the solution  $2\frac{1}{2}$  liters of ether are added. A gummy substance is precipitated, which, after decantation of the supernatant liquid, is dissolved in 50 cc. of water and enough alcohol to make 1 liter, the alcohol being slowly added and thoroughly mixed with the syrup. The solution is precipitated again with  $2\frac{1}{2}$  liters of ether. The liquid is decanted from the precipitate and the two ethereal extracts mixed and allowed to stand over night during which time they become clear. The clear solution is poured off from the gummy substance which settles out, the ether is recovered by distilling on a steam bath, and the remaining alcoholic solution is evaporated under diminished pressure to a thick syrup. This is removed from the flask with an equal volume of alcohol. To the solution ether is then added. Upon scratching the flask, or quicker by seeding, the rhamnose separates out, requiring generally a day for complete crystallization. The sugar is filtered off as dry as possible on a Büchner funnel, washed first with a mixture of 1 part ether and 2 parts alcohol, then with a 1:1 alcohol and ether mixture, and finally with ether.

The yield is from 50 to 55 gm. of white sugar. It may be recrystallized from water or alcohol in the usual way.

## BIOCHEMISTRY OF BACILLUS ACETOETHYLICUM WITH REFERENCE TO THE FORMATION OF ACETONE.\*

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Several microorganisms<sup>1</sup> have been described as forming more or less acetone, and at least two<sup>2</sup> have been used on a commercial scale. Of these organisms, *Bacillus macerans* seemed the most desirable for the present work, inasmuch as it forms ethyl alcohol as a by-product. Since it was not possible to obtain a culture of the organism in this country, experiments were undertaken with the view of isolating the bacillus.

### I. Isolation of *Bacillus*.

Samples of potatoes were obtained from various parts of the country. Cylinders were cut from these, put in test-tubes, and sterilized for 20 minutes in the Arnold. The tubes were then in-

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The present work was undertaken at the suggestion of the Council of National Defense in an attempt to develop a fermentation process for the production of acetone. Attention, therefore, has been centered on the acetone formation so that the work is incomplete in many other respects.

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§ Lt. Senior collaborated in the early part of the work and developed the analytical methods used.

<sup>1</sup> Kayser, E., *Ann. Inst. Pasteur*, 1894, viii, 737. Bréaudat, L., *Ann. Inst. Pasteur*, 1906, xx, 874. Schardinger, F., *Wien. klin. Woch.*, 1904, xvii, 207; *entr. Bakteriolog., 2te Abt.*, 1905, xiv, 772.

<sup>2</sup> F. Bayer and Co., *D.R.P.* 283,107, July, 1913; 291,162, Jan., 1914; *British Pat.* 14,371, June, 1914. Delbrück, K., and Meisenburg, K., *U. S. Pat.* 1,69,321. Fernbach, A., and Strange, E. H., *U. S. Pat.* 1,044,368; 1,044,446; 1,044,447.

cubated. After several days one set of tubes containing potatoes from Berkshire County, Massachusetts, showed gas formation and crumbling somewhat as described by Schardinger.<sup>3</sup> The fermented potatoes from these tubes were distilled with a little water and the distillate was tested for acetone with paranitrophenylhydrazine. A strong positive test was obtained.

Microscopic examination of the culture showed the presence of large oval spores and many long thin rods. Some of the material was plated on 2 per cent glucose agar and gave small translucent colonies. Several of these were replated three times and then transferred to glucose agar slants. The culture was kept growing on this medium as it was found that the fermentative power remained constant under these conditions. Potato medium inoculated from these slants gave consistently positive tests for acetone. An organism was therefore at hand in pure culture which produced acetone from starch. The general cultural characteristics of the organism were studied and are summarized in Table I, in accordance with the chart of the Society of American Bacteriologists. The fermentation reactions and the experiments on the optimum conditions for the fermentation will be described in more detail below.

TABLE I.

*Description of the Organism.*

Described according to descriptive chart of the Society of American Bacteriologists.

**Source.** From old potatoes obtained from Berkshire Co., Mass., July 1, 1917.

**Proposed name.** *Bacillus acetoethylicum*.

**I. MORPHOLOGY.****1. Vegetative Cells.**

Motile.

*From 24 hr. agar slant, 40°C.*

Short rods 4 to 6 $\mu$   $\times$  0.2 to 0.3 $\mu$ .

No chains.

Ends rounded. Stain evenly with Loeffler's methylene blue or gentian violet. Gram-negative.

*From 24 hr. 10 per cent corn media.*

Same as above but occasional short chains.

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<sup>3</sup> Schardinger, F., *Wien. klin. Woch.*, 1904, xvii, 207.

TABLE I—*Continued.*

*From old (6 to 10 days) 10 per cent corn media.*

Stain unevenly with deeply staining spot at end or in center.

2. **Spores**, elliptical, form at end of rods. Stain easily with methylene blue or gentian violet. 0.5 to 1.0 $\mu$  in diameter.

## II. CULTURAL FEATURES.

1. **2 Per Cent Glucose Agar Slant.**

24 hrs., 40°C. Moderate, spreading, effuse, dull, translucent, no odor. Shiny.

*Condensation water opaque.*

2. **Potato.**

24 hrs., 40°C. Gas bubbles all over medium, crumbles easily, no odor.

2 to 3 days, 40°C. Medium sinks to greyish white paste.

3. **Glucose Broth.**

24 hrs., 40°C. Cloudy, no odor.

2 to 3 days. Slimy mass in bottom.

4. **Litmus Milk.**

24 hrs., 40°C. Bottom of tube white, no gas, odor, acid, or clot.

36 hrs., 40°C. Milk red on top, rest white.

72 hrs., 40°C. Same but coagulated; clot does not digest subsequently.

5. **Agar Plate Colonies.**

2 per cent glucose agar, 24 hrs., 40°C.

Growth slowly spreading.

Round, outline irregular.

Surface smooth.

Elevation effuse.

Edge entire or undulate.

Internal structure coarsely granular.

6. **Sodium Chloride in Bouillon.**

Inhibiting concentration 4 to 5 per cent.

7. **Nitrogen.**

*With sugar as carbohydrates* obtained from peptone, proteins, or ammonium salts.

*With starch*, same but cannot use ammonium salts.

8. **Best Medium for Long Continued Growth.**

2 per cent corn in media with CaCO<sub>3</sub>.

## III. PHYSICAL AND BIOCHEMICAL FEATURES.

1. **Optimum Reaction of Media.**

For growth, pH = 8.0 to 9.0.

For fermentation, pH = 6.0 to 8.0.

2. **Vitality on Culture Media.**

At least 6 months at room temperature.

At least 1 month at 40°C.

3. **Temperature Relation.**

Optimum temperature 40–43°C.

Spores may be boiled at least 20 minutes.



TABLE I—*Concluded.***4. Resistant to Drying.****5. Products of Reaction.**

Formic acid.

Ethyl, propyl, butyl alcohol.

Acetone.

**6. Fermentation of Sugars, Etc.**

Ferments the following sugars in 1 per cent solution with addition of  $\text{CaCO}_3$  and peptone as nitrogen source, 15 cc. in test-tubes.

Ferments: levulose and galactose under following conditions.<sup>4</sup>

Medium: 1 gm. of  $\text{KH}_2\text{PO}_4$ , 1 gm. of  $(\text{NH}_4)_2\text{HPO}_4$ , 0.01 gm. of NaCl, 1.0 gm. of  $\text{CaCO}_3$ , 10.0 gm. of levulose, per liter. Put in tubes and sterilized as described by Schardinger; inoculated with pure culture of the bacteria and incubated 12 days at 40°C.

Acetone = 8 to 9 per cent.

Alcohol = 14 to 20 per cent.

Starch does not ferment under these conditions.

*Fermentation Reactions.*

Temperature 37°. Time 10 days. Medium: 2 per cent sugar, 0.5 per cent peptone, 2 per cent  $\text{CaCO}_3$ .

Substance.	Acetone.	Alcohol.	Substance.	Acetone.	Alcohol.
	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>
Galactose.....	4- 5	22-24	Dextrin.....	6- 7	14-16
Maltose.....	6- 7	23-24	Dextrose.....	9-10	22-23
Mannose.....	6- 7	22-23	Levulose.....	8-10	24-25
Raffinose.....	8-10	22-23	Xylose.....	4- 5	18-20
<i>d</i> -Arabinose.....	6- 7	12-16	Glycerol.....	—	40-43
Ca lactate.....	—	—	Sucrose.....	8- 9	24-26
Starch.....	8-10	20-24			

**7. Air Relation.**

Facultative anaerobe.

**8. Slime Formation.**

In 10 per cent sugar solutions, having a reaction of pH 8.0 to 9.0, large quantities of slime are formed so that the whole medium becomes very viscous. Under conditions of fermentation a small deposit of slime settles to the bottom.

**IV. PATHOGENICITY****1. Non-Pathogenic to Mice.**

<sup>4</sup> Schardinger, F., *Centr. Bakteriöl.*, 2te Abt., 1905, xiv, 772.

The organism resembles *Bacillus macerans* therefore in its cultural characteristics in so far as can be determined from the rather brief description given by Schardinger.<sup>4</sup> It differs from it, however, in that it ferments galactose and levulose under anaerobic conditions with ammonium salts as the source of nitrogen. This distinction seems sufficient to differentiate the organism from *Bacillus macerans*. The name *Bacillus acetoethylicum* is suggested as it indicates the formation of acetone and ethyl alcohol, the most striking characteristic of the organism.

## II. Identification of the Products of Reaction.

*Culture medium.*—10 gm. of peptone, 100 gm. of potato starch, and 20 gm. of finely divided calcium carbonate were boiled with 2 liters of water in a large Florence flask. The flask was plugged with cotton and autoclaved for 3 hours. It was inoculated with the growth from one glucose agar slant of the organism and the cotton plug tightly covered with tin-foil to prevent evaporation.<sup>5</sup> The culture was then incubated at 40°C. for 14 days, made alkaline with sodium hydroxide, and distilled.

*Acetone.*—The presence of acetone was established by the formation of characteristic needles with paranitrophenylhydrazine, by the iodoform test, and by precipitation with mercuric sulfate.

*Alcohol.*—20 cc. of distillate were made up to 200 cc. and oxidized with potassium dichromate as follows.

50 cc. of diluted distillate, 100 cc. of 1:1 sulfuric acid (100 cc. of acid made up to 200 cc. with water), and 75 cc. of 2 N  $K_2Cr_2O_7$  were put in a 500 cc. pressure bottle. Three other similar bottles were prepared. They were heated for 15 minutes on the boiling water bath and the contents of the four bottles were combined, cooled, and made alkaline with NaOH. 300 cc. were then distilled off, and the residue was made acid with sulfuric acid and slowly distilled until the distillate was only faintly acid. The distillate was neutralized to litmus with NaOH and evaporated to small volume on the steam bath. It was then acidified with sulfuric acid and extracted five times with an equal volume of ether, and the ether extract combined and filtered. The filtrate was made alkaline with am-

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<sup>5</sup> This is necessary since it was found that if the flask was merely plugged with cotton only traces of acetone would be found after fermentation. That this is due to evaporation is shown by the fact that if the escaping gas from the flask is allowed to bubble through water a positive test for acetone is obtained from the latter.

monia and allowed to evaporate at room temperature. A concentrated solution of silver nitrate was added slowly and the resultant precipitate collected in three portions, dried, and analyzed for silver.

The results are shown in Table II.

The last fraction was therefore probably nearly pure silver acetate, while the first two fractions must have contained more or less of the higher salts.

*Acid.*—The residue from the above distillation was made acid with phosphoric acid and steam-distilled until 1 liter of distillate had collected. This was made alkaline with ammonia and evaporated to 100 cc. It was made slightly acid with sulfuric acid and extracted four times with an equal volume of ether.

*Ether Extract.*—The ethereal extract was made slightly alkaline with ammonia and allowed to evaporate at room temperature. The residue gave no precipitate

TABLE II.  
*Analysis of Silver Salts.*

Fraction No.	Weight of salt.	Weight of silver.	Per cent of silver.
	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
1	0.1106	0.0663	59.95
2	0.1012	0.0635	62.87
3	0.1023	0.0658	64.32
Calculated for: Silver acetate.....			64.64
" propionate.....			59.64
" butyrate.....			55.35

with silver nitrate, showing the absence of any appreciable quantity of the higher acids.

*Water Solution.*—The water solution after extraction with ether was made alkaline with ammonia and evaporated to small volume. The addition of silver nitrate caused the formation of a silver mirror and a precipitate of metallic silver. The principal acid formed is therefore formic.

Acetone, ethyl alcohol, and formic acid are therefore the principal non-gaseous products of fermentation.

### *III. Effect of Varying Conditions on the Yield of Acetone.*

The course of any fermentation may be considered as a function of the following variables: physiological state of the culture, tem-

perature, air supply, reaction of the media, and the chemical and physical properties of the substrate. The effect of varying these factors on the yield of acetone was studied with a view of determining the optimum conditions for its formation.

In order to carry out these experiments it was first necessary to have a convenient quantitative method for the determination of acetone and alcohol.

*Determination of Acetone.*—The method used was a modification of the Messinger-Huppert<sup>6</sup> method and was carried out as follows. An aliquot portion of the fermented media, containing 50 to 150 mg. of acetone, was diluted to 120 cc. with water, made alkaline with NaOH, and 40 cc. were distilled over into a 100 cc. volumetric flask. 40 cc. of water were placed in this flask before beginning the distillation and the delivery tube from the condenser allowed to project below the level of the water—this is necessary in order to prevent loss by evaporation. After 40 cc. had been collected, the distillate was made up to 100 cc. and 10 cc. were pipetted into a glass-stoppered bottle, 10 cc. of 10 per cent NaOH added, and an excess of 0.106 N iodine in KI was run in with constant agitation. The bottle was stoppered and allowed to stand 2 minutes. It was then made acid by the addition of 2 cc. of 1:1 sulfuric acid and the excess iodine titrated back with thiosulfate. This back titration should approximately equal the amount of iodine actually used in the determination.

The iodine solution was standardized against a solution of acetone made up by weight and containing twice the amount of alcohol, since it was found that this is approximately the ratio in which the two substances are formed. The correction for alcohol (which is 0.02 to 0.03 cc. per mg. of alcohol) is thus made automatically in the standardization. It was found under these conditions that 1 cc. of 0.1065 N iodine was equivalent to 1 mg. of acetone.

The accuracy of the method was checked by making control determinations with Van Slyke's<sup>7</sup> gravimetric mercury sulfate method. The agreement was always within 2 per cent which was more than sufficient for the purposes of this investigation.

*Alcohol Determination.*<sup>8</sup>—10 cc. of distillate containing less than 20 mg. of alcohol were pipetted into a magnesia bottle containing 15 cc. of 0.2 N potassium dichromate, 20 cc. of 1:1 sulfuric acid were added, and the stopper was clamped in. The bottle was then warmed and finally heated in the boiling water bath for 45 minutes. After cooling it was opened, the solution diluted to 150 cc., 10 cc. of 10 per cent KI were added, and the liberated iodine was titrated

<sup>6</sup> Messinger, J., *Ber. chem. Ges.*, 1888, xxi, 3366. Rakshit, J. N., *Analyst*, 1916, xli, 245.

<sup>7</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxxii, 455.

<sup>8</sup> Dupré, *J. Chem. Soc.*, xx, 495.



with thiosulfate, using starch as an indicator. A slight correction (0.03 cc. of 0.1 N iodine per mg.) is necessary for the acetone present and a correction of 0.15 to 0.30 cc. for the reagents. The solutions were standardized against solutions of alcohol made up by weight.

It was now possible to follow the changes in the production of acetone and alcohol.

*Temperature.*—In order to determine the optimum temperature for fermentation a series of cultures was made up and placed in incubators set at 36, 43, and 46°C. respectively.

Since the effect of the reaction, etc., was as yet undetermined, the cultures were made in what were considered the best conditions.

TABLE III.

*Effect of Temperature.*

Time of fermentation 15 days.

Medium: 2 per cent potato flour, 1 per cent peptone, 2 per cent  $\text{CaCO}_3$ .

Experiment No.	Acetone, in per cent of weight of starch.		
	36°C.	43°C.	46°C.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	5.7	8.4	7.7
2	7.1	8.8	7.0
3	6.5	8.6	7.5

*Medium.*—20 gm. of potato flour were mixed with a little cold water and poured with stirring into 700 to 800 cc. of boiling water containing 10 gm. of peptone. The suspension was boiled, 20 gm. of precipitated calcium carbonate were added, and the solution was made up to 1 liter. 50 cc. were measured into 120 cc. Erlenmeyer flasks which were plugged with cotton and sterilized 1 hour in the autoclave. They were then cooled and inoculated from a 24 hour glucose agar slant of the organism. The cotton plugs were tightly covered with tin-foil to prevent evaporation. In this and in all the other experiments the cultures were examined microscopically after incubation and any showing contaminating organisms were discarded. It was found that most of the common contaminating organisms destroy the acetone formed.

The results of the experiment are shown in Table III. It will be seen that the optimum temperature is about 43°C. and also that this is not due merely to the rate of fermentation since the cultures were left in the incubator 5 or 6 days after all signs of fermentation had ceased.

*IV. Influence of the Reaction of the Media.*

The study of the reaction of the media on the fermentation is complicated by the fact that, in order to maintain the reaction even approximately constant, it is necessary to use relatively high concentrations of buffer substances which may have a secondary effect on the fermentation. In order to reduce the amount of acid formed, a low concentration of carbohydrate was used. It is also necessary to note that the concentration of buffer is higher in the more alkaline cultures.

*Cultures 1 to 10.*—20 gm. of soluble starch, 5 gm. of peptone, and 34 gm. of  $\text{Na}_2\text{HPO}_4$  were dissolved in 2 liters of water. 100 cc. of this solution were then pipetted into a series of 150 cc. Erlenmeyer flasks and titrated to the desired reaction with 0.25 M NaOH. After sterilization the reaction was measured colorimetrically, using Sørensen's standard solutions with bromocresol purple and phenol red.<sup>9</sup>

*Cultures 11 and 12.*—These cultures contained an excess of solid  $\text{MgCO}_3$  in place of the phosphate. After sterilization the reaction was strongly alkaline to phenolphthalein and remained so throughout the fermentation.

The results are tabulated in Table IV. The optimum reaction for the formation of acetone under these conditions is therefore between pH 6.0 and 7.0. It was found that precipitated  $\text{CaCO}_3$  would maintain the reaction at about this point and it was therefore used in the later fermentations.

It is interesting to note that in Cultures 11 and 12, which had a decidedly alkaline reaction, there was a very heavy growth of the organism but very little acetone formed. The optimum reaction for growth must therefore be on the alkaline side of the optimum for the production of acetone. Attempts were made to decide this question quantitatively by plating the cultures and counting the number of organisms. The results were very unsatisfactory, however, due to the fact that the organism collects in slimy masses which prevent accurate dilution. It was found, however, that no growth could be obtained on agar titrated to pH 6.4, although this is approximately the optimum for the production of acetone. These results seem to justify the conclusion that there are different optimum

<sup>9</sup> Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1.

reactions for the different activities of the organism and possibly of bacteria in general. It was found later while working with cane molasses, which is rather unfavorable as a culture medium, that no growth could be obtained if the reaction, to begin with, was more acid than pH 6.8, while a vigorous fermentation took place if the reaction was first adjusted to pH 8 to 9. This is due to the fact that the organism does not grow in the molasses except under the most

TABLE IV.

*Effect of Reaction.*

Temperature 42°C. Time of fermentation 11 days.

Media: Cultures 1-10, 1 per cent soluble starch, 0.25 per cent peptone, 1.7 per cent  $\text{Na}_2\text{HPO}_4$ .

Cultures 11-12, 1 per cent soluble starch, 0.25 per cent peptone, excess solid  $\text{MgCO}_3$ .

Titrated to reaction noted with 0.25 M NaOH.

	Culture No.											
	1	2	3	4	5	6	7	8	9	10	11	12
Reaction of media after sterilization, pH.....	5.0	6.0	6.0	6.4	6.4	6.8	6.8	7.2	7.2	7.6	10	10
Reaction of media after fermentation, pH.....	5.0	5.6	5.8	6.0	6.2	5.8	6.1	6.4	6.6	6.6	10	10
Acetone, per cent.....	—	8.9	8.6	9.2	10.0	6.6	6.8	6.4	4.4	6.6	Trace.	
Alcohol, per cent.....	No growth.	22.0	22.0	23.6	23.6			19.0		21.0	Heavy growth, very viscous.	

favorable conditions and hence when the reaction is too acid no growth takes place. When the media are more alkaline, however, (pH 8 to 9) and therefore near the optimum for growth, the organism multiplies rapidly and at the same time produces sufficient acid to change the reaction to pH 6.0 to 7.0, where it is maintained by the carbonate present. This is about the optimum for acetone production. Table V shows the results of such a series. It will be seen that the reaction of the culture which had been corrected to

pH 9.0 to 9.5 was changed by the acid produced to pH 6.6 to 6.8 in the course of the first 24 hours.

TABLE V.

*Effect of Reaction of Medium.*

Temperature 42°C.

Medium: 5 cc. of cane molasses, 100 cc. of water, 2 gm. of  $\text{CaCO}_3$ , titrated with NaOH to reaction noted.

Time after inoculation.	Culture No.			
	1	2	3	4
pH of media.				
<i>days</i>				
0	6.6	6.8	9.5	9.3
1	6.6	6.8	7.0	6.8
6	6.6	6.7	6.6	6.4
Per cent of sugar as acetone.				
6	No fermentation.		8.5	9.0

*V. Physiological Condition of the Culture.*

It is well known that the character of the fermentation caused by some microorganisms may be decidedly changed according to the age and condition of the culture used for inoculation. In order to see if there was any such effect with *Bacillus acetoethylicum* a series of cultures was inoculated from the same parent culture at different times.

The data for this experiment are given in Table VI. It is evident that there is no marked effect upon the yield of acetone due to the age of the inoculating culture. It is necessary to note that the parent cultures were not tightly stoppered and that the volatile products of reaction therefore evaporated. This is shown by the fact that only 2 to 3 per cent of acetone was found in the parent flasks.

A second series of cultures was now made in which the flasks were tightly covered with tin-foil to prevent evaporation.



TABLE VI.

*Effect of Age of Culture.*

Media: Parent culture, 1,000 cc. of H<sub>2</sub>O, 50 gm. of corn-meal, 20 gm. of CaCO<sub>3</sub>, pH 7.6. Temperature 42°C.

Subculture, 50 cc. of H<sub>2</sub>O, 5 gm. of corn-meal, 1 gm. of CaCO<sub>3</sub>, 0.25 gm. of peptone. Incubation 11 days. Temperature 43°C.

Time after which subculture was inoculated.	Per cent of corn as acetone in subculture.		Microscopic appearance of parent culture.
	A	B	
<i>days</i>	<i>per cent</i>	<i>per cent</i>	
1	7.8	7.6	Rods.
2	7.9	7.1	"
3	8.0	8.4	"
4	8.0	8.5	" Few spores.
5	8.4	8.0	Mostly spores.
7	7.7	8.3	" "
9	7.8	6.4	" "
16	7.8	8.9	All spores.
30	7.15	6.8	" "
150	7.9	7.4	" "
Parent culture analyzed after 14 days.....	2.8	2.4	

TABLE VII.

*Effect of Successive Inoculation from Fermented Cultures.*

Medium: 2.5 gm. of corn-meal, 0.5 gm. of peptone, 2 gm. of CaCO<sub>3</sub>, 50 cc. of water. Temperature 42°C.

Series.	Time of fermentation.	Acetone in flask No.				
		1	2	3	4	5
	<i>days</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
A.	7	8.0	8.5	8.1	9.0	9.3
B, inoculated after 7 days from corresponding number in Series A.	7	5.1	5.3	3.8	4.0	3.4
C, inoculated after 7 days from corresponding number in Series B.	7	3.3	3.8	2.5	3.8	2.1

2.5 gm. of corn-meal, 0.5 gm. of peptone, 2 gm. of  $\text{CaCO}_3$ , and 50 cc. of water were sterilized in 120 cc. Erlenmeyer flasks. The flasks were inoculated and tightly covered with tin-foil. They were incubated 8 days. A second series of cultures prepared in the same way was then inoculated from these and the remainder of the solution analyzed.

The results are tabulated in Table VII. The yield of acetone is lower in each succeeding generation showing that the fermentative power of the organism is injured under these conditions. Since this was not the case in the preceding experiments it seems probable that the injury is due to some volatile product of fermentation. If the fermentation is carried out in closed vessels, therefore, it is not possible to use a culture which has undergone complete fermentation for the inoculation of fresh media. It will be shown in the experiments on a semicontinuous fermentation, however, that this may be done under certain conditions; *i.e.*, a shorter time of fermentation.

#### VI. Rate of Formation of Acetone and Alcohol.

In the preceding experiments the cultures were left in the incubator until all signs of fermentation had ceased. In order to deter-

TABLE VIII.

#### Rate of Formation of Acetone and Alcohol.

Medium: 10 gm. of potato starch, 4 gm. of peptone, 10 gm. of  $\text{CaCO}_3$ , 500 cc. of water. Temperature 42°C.

Time analyzed as noted.

Time after in- oculation.	Culture No.						Average.		Ratio alcohol to acetone.	
	1		2		3					
	Acetone.	Alcohol.	Acetone.	Alcohol.	Acetone.	Alcohol.	Acetone.	Alcohol.		
	days	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	
3	4.6	12.3	4.0	11.4	3.3	10.7	3.9	11.5	2.9	3.65
4	7.4	16.1	5.6	13.5	4.6	12.3	5.9	13.9	2.3	2.9
5	8.9	18.1	7.4	16.6	6.1	14.7	7.5	16.5	2.2	2.8
6	9.2	18.9	8.6	18.4	7.4	18.0	8.4	18.4	2.2	2.8
7	9.3	19.4	9.0	19.4	7.7	18.1	8.7	18.9	2.2	2.8
9	9.3	19.5	9.1	19.4	8.3	20.0	8.9	19.6	2.2	2.8
13	9.4	19.5	9.0	19.3	8.8	20.2	8.7	19.6	2.2	2.8

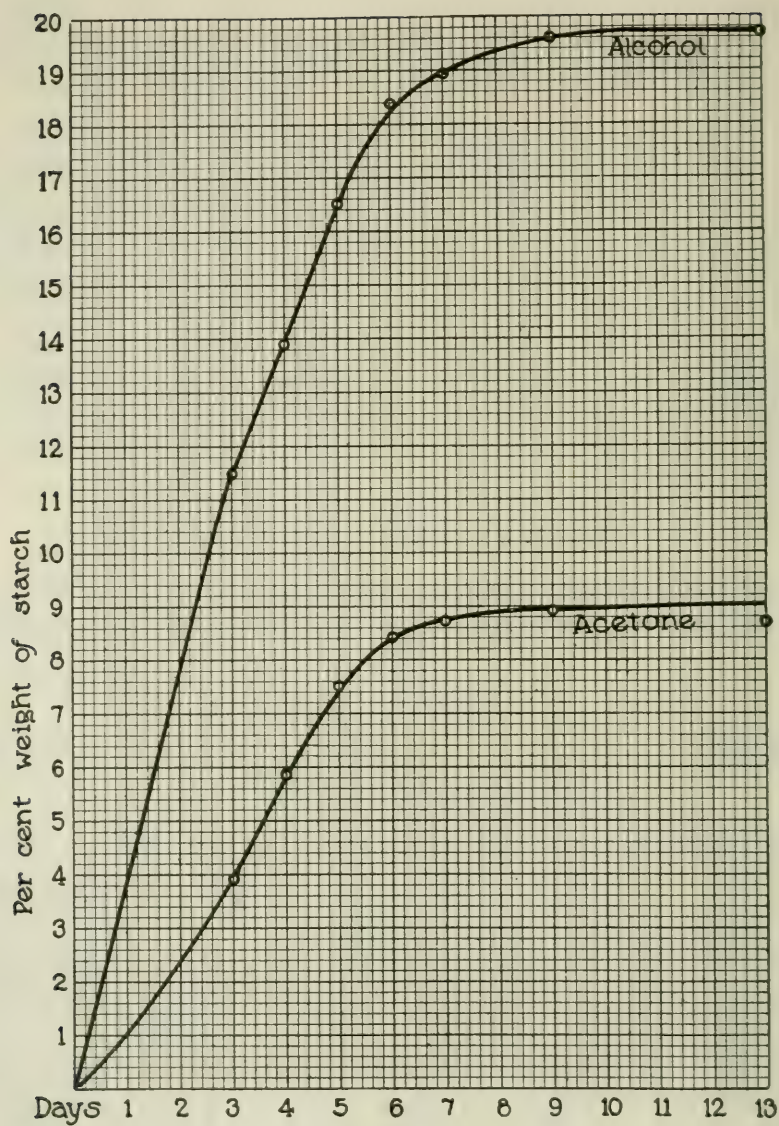


FIG. 1.

mine the rate of formation of the products three cultures were inoculated and analyzed at intervals.

500 cc. of water, 10 gm. of potato starch, 4 gm. of peptone, and 10 gm. of  $\text{CaCO}_3$  were boiled in a 1 liter Pasteur flask. The flask was autoclaved 1 hour, inoculated, and incubated at  $42^\circ$ . Two similar flasks were prepared. Samples were withdrawn and analyzed for acetone as shown in Table VIII and in Fig. 1. Unfortunately the analyses were not made until the third day, so that the first

TABLE IX.

*Effect of Size of Inoculation.*

Medium: 5 gm. of corn-meal, 2 gm. of  $\text{CaCO}_3$ , 0.5 gm. of peptone, 50 cc. of  $\text{H}_2\text{O}$  in 120 cc. flasks. Temperature  $42^\circ\text{C}$ .

Inoculation.	Per cent of corn as acetone.					
	Flask No.	After 5 days.	Flask No.	After 7 days.	Flask No.	After 10 days.
		<i>per cent</i>		<i>per cent</i>		<i>per cent</i>
1     slant each.	1	6.4	4	7.1	7	8.5
	2	6.4	5	7.6	8	8.7
	3	6.2	6	8.0	9	8.1
0.1    "    "	10	7.0	13	7.4	16	7.9
	11	7.0	14	7.5	17	8.0
	12	6.0	15	7.4	18	9.4
0.01   "    "	19	6.4	22	7.1	25	8.9
	20	6.4	23	7.4	26	8.3
	21	6.9	24	7.4	27	8.2
0.001   "    "	28	6.5	31	7.3	34	8.2
	29	5.6	32	6.8	35	8.1
	30	5.6	33	6.2	36	7.8

part of the curve is missing. It will be seen, however, that the ratio of alcohol to acetone decreases from 2.9 to 2.2 (by weight) where it remains constant. The molecular ratios were calculated on the assumption that the alcohol formed is all ethyl alcohol—this is not strictly true.

Under the conditions of the preceding experiment the fermentation requires 7 to 9 days. It seemed possible that this time might be shortened by using a larger inoculation. This was tested as follows.



A series of flasks was prepared containing 5 gm. of corn-meal, 2 gm. of  $\text{CaCO}_3$ , 0.5 gm. of peptone, and 50 cc. of water. They were sterilized, inoculated with varying amounts of the organism, incubated, and analyzed as usual.

*Inoculation.*—The growth from 10 agar slants was washed off in 10 cc. of sterile salt solution. Flasks 1 to 9 were then inoculated with 1 cc. of this suspension, equivalent to the growth from one slant; Flasks 10 to 18 with 1 cc. of a 1:10 dilution, etc.

The results of the series are shown in Table IX. The size of the inoculation, within the limits of this experiment, evidently has very little effect on the time required for the completion of the fermentation. It was noted that fermentation commenced a little sooner with the heavier inoculation but since about the same time was required to complete the fermentation little is gained.

### *VII. Fermentation of Various Sugars.*

The optimum conditions for the fermentation being known, it was now possible to determine the yields produced by the various sugars. The results of these experiments have been given in Table I. The organism ferments pentoses as well as hexoses and starch. No acetone is formed from glycerol which is transformed very largely into alcohol.

### *VIII. Experiments on Corn, Molasses, Etc.*

The cheapest sources of starch or sugar are corn, molasses, and potatoes. A series of fermentations was therefore run to determine which of these substances would furnish the best yield.

The results of this series of experiments are summarized in Table X. It appears from these results that corn is the most favorable material for fermentation commercially. Experiments were therefore undertaken to see if the yield could be increased.

The influence of the following factors was studied: (1) addition of nitrogenous material; (2) effect of varying the air supply; and (3) concentration.

The results of the experiments on the addition of nitrogenous material and on the effect of the air supply are summarized in Table XI. The addition of nitrogenous material as peptone or yeast evi-

dently increases the acetone yield considerably when little air is admitted to the culture. Aerobic conditions cause the same increase in the yield of acetone with, however, a decrease in the yield of alcohol. Addition of nitrogen under these conditions causes no further

TABLE X.

*Yield of Acetone from Various Raw Materials.*

Temperature 42°C. Time of fermentation 10 days.

	Substrate.			
	Corn (per cent of dry weight).	Potatoes (per cent of fresh weight).	Cane molasses (per cent of weight of total sugar content).	Beet molasses (per cent of weight of total sugar content).
Acetone.....	6.1	2.6	9.0	9.5
" ".....	6.0	2.5	10.1	10.5
Alcohol.....	13.5	6.0	24.7	26.1

TABLE XI.

*Effect of Air and Nitrogen on Yield from Corn.*

Temperature 42°C. Time of fermentation 8 days.

Medium: 2 gm. of corn-meal, 50 cc. of water, 2 gm. of  $\text{CaCO}_3$  in different size flasks and with peptone added as noted.

	Size of flasks.					
	120 cc.	120 cc.	120 cc.	500 cc.	500 cc.	500 cc.
Peptone added, gm.....	0	0.5	1.0	0	1.0	1.0
Acetone yield (per cent of corn).....	5.9	9.4	9.0			
" " " " " ".....	5.9	8.1	8.3	10.5	11.0	10.1
" " " " " ".....	6.1	9.5	9.6	11.5	10.0	9.8
Alcohol " " " " " ".....	15.0	20.1	21.0	15.6	17.9	22.0
" " " " " ".....				17.8	18.0	21.2

increase in the amount of acetone produced. This indicates either, first, that the organism requires less nitrogen under aerobic conditions, or second, that it is able to use some of the nitrogenous material present in the corn which it cannot use in the absence of air. The latter explanation is probably correct.

It will be noted that the corn yields about the same percentage of acetone as does corn-starch; *i.e.*, it ferments as though it were all starch whereas it contains not over 60 per cent. There is evidently some condition changed which causes the organism to produce a higher yield of acetone from starch present in the corn than it does from purified corn-starch. It seemed possible that this might be due to the presence of inert material. Many substances, such as cotton, asbestos, filter paper, etc., were tried but with no noticeable result. The addition of corn protein to the purified corn-starch was also without effect.

*Concentration.*—The results of a series of cultures of increasing concentration are summarized in Table XII. The maximum con-

TABLE XII.

*Concentration of Corn.*

Temperature 42°. Time of fermentation 12 days.

Medium: 50 cc. of water, 2 gm. of  $\text{CaCO}_3$ , 1 gm. of peptone in 120 cc. Erlenmeyer flasks with amount of corn-meal as noted.

Acetone (in per cent of corn).				
2.5 gm. of corn-meal.	3.25 gm. of corn-meal.	5.0 gm. of corn-meal.	7.5 gm. of corn-meal.	10.0 gm. of corn-meal.
9.0	9.0	8.3	5.6	5.0
9.3	8.9	7.7	5.4	5.0

centration which will completely ferment is about 8 parts of corn to 100 of water.

*Experiments with Molasses.*—The results in Table X show that both cane and beet molasses can be fermented with fairly good yields. Several experiments on the addition of peptone, phosphates, etc., were made, without however causing any increase in the yield.

Cane molasses is usually neutral or slightly acid and requires the addition of small amounts of alkali in order to bring it to the proper reaction.

The main disadvantages from a manufacturing standpoint of the fermentation as carried out in the preceding experiments is the long time required for fermentation and the difficulty of keeping the culture sterile.

It was noted in the cultures which had undergone fermentation that the calcium carbonate was held together by a thick viscous slime. Smears made from this material showed the presence of large numbers of the organism whereas smears made from the supernatant liquid showed comparatively few. It seemed probable, therefore, that if some inert material could be added to the culture so as to retain this slime and allow the clear liquid to drain off, most of the organisms would be retained and could be used to start the succeeding fermentation. Experiments showed that this could be done. The fermentation was now semicontinuous and considerably more rapid. Owing to the clogging up of the cultures when corn was used these experiments were carried out with molasses. The cultures were grown in 500 cc. Florence flasks with a small piece of tubing fused to one side as in a Pasteur flask. The mouth of the flask was plugged with cotton and covered with tin-foil and the side arm was closed by a piece of rubber tubing and a glass plug. The flasks were manipulated for refilling, etc., in the same way as an ordinary Pasteur flask. Almost any inert material such as corn cobs, coke, branches, etc., could be used to fill the flasks. In the experiments tabulated in Table XIII, branches were used. The flasks were filled with broken twigs, diluted molasses was poured in, and the flasks were plugged, sterilized, and inoculated as usual. At the times stated in the table the fermented medium was poured out and fresh sterile medium put in from a similar flask.

It will be seen that, under these conditions, the fermentation is complete in 48 hours and that the residue from an old fermentation is able to start a new one. The medium does not become sufficiently acid to stop the fermentation even though no neutralizing material is present. This is due partly to the fact that the fresh medium is made alkaline (pH 8 to 9). This, however, is not the complete explanation since the same medium when inoculated fresh from an agar slant develops acid and ceases to ferment. Controls without any inert material fermented slowly and soon stopped producing acetone. On the other hand, fresh cultures inoculated with a large volume (20 per cent) of the old fermented medium ferment slowly and usually show contaminating organisms. Smears made from the continuous cultures occasionally show contami-



nating organisms also. These contaminations, however, did not affect the production of acetone as a rule, and often disappeared after one or two refillings.

In these experiments each succeeding fermentation is inoculated from the preceding one without any lowering of the yield. In the experiments described in Table VII it was found that this procedure resulted in lower and lower yields. The difference is due to the

TABLE XIII.

*Semicontinuous Fermentation.*

Temperature 42°C. Time of fermentation 48 hrs.

Medium: 500 cc. Florence flasks with side necks, filled with broken twigs. 6.5 per cent beet molasses (by volume) containing 70 mg. of total sugar per cc. pH = 8.0 to 9.0. Inoculation, 1 slant each. Emptied and refilled every 48 hours.

Fermentation No.	Per cent of sugar as acetone in culture No.			
	1	2	3	4 (no twigs).
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	3.3	2.15	3.1	3.0
2	8.1	3.75	4.5	4.2
3	7.8	10.2	6.5	2.6
4	7.0	7.5	8.1	2.0
5	7.7	8.1	9.4	1.1
6	9.6	9.3	8.2	Trace.
7	7.2	9.6	8.1	
8	9.0	9.0	8.2	
9	6.8	8.4	8.7	
10	8.4	8.5	7.9	
11	8.1		9.2	
12	8.0			
13	8.2			

fact that in the semicontinuous cultures just described the fermentation is complete in 40 to 50 hours whereas in the first series 7 days are required. This explanation is borne out by the fact that, if the cultures are refilled every 5 or 6 days instead of every 48 hours, the yields steadily decrease in the same way as was found in the early experiments.

These results show that the time of fermentation can be very materially shortened under these conditions and that the danger of

contamination is also lessened. Attempts were made to make the process more nearly continuous by adding the fresh mash to the bottom of the fermenting culture and allowing the fermented mash to flow off at the top. They were unsuccessful, however, due to the difficulty of handling such an apparatus on a laboratory scale. This method was used successfully, however, in a large fermenter of 4,000 liters capacity. These experiments will be described elsewhere.<sup>10</sup>

#### SUMMARY.

1. An organism has been described which produces acetone and ethyl alcohol with smaller amounts of higher alcohols from starch or sugar.
2. The optimum conditions of temperature, reaction of the media, physiological state of the culture, size of inoculation, air supply, and nitrogen supply have been determined.
3. A semicontinuous method for carrying on the fermentation has been described.

The authors wish to acknowledge their indebtedness to Dr. P. A. Levene for many helpful suggestions during the course of the work.

<sup>10</sup> Northrop, J. H., Ashe, L. H., and Morgan, R. R., *J. Ind. and Eng. Chem.*, 1919, xi, 723.



## EPICHITOSAMINE AND EPICHITOSE.

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(Received for publication, June 30, 1919.)

Only two  $\alpha$ -aminohexoses have been isolated in form of their simple salts: namely, chitosamine and chondrosamine. Xylohexosamine has been prepared only in form of its benzoyl derivative. These three sugars present the entire list of nitrogenous sugars prepared synthetically. The number of them found in nature is limited to two. In the present communication account is given of the synthesis of epichitosamine. This sugar and chitosamine will constitute the first known pair of epimeric hexosamines. Although interesting in itself the sugar was prepared principally for one definite reason. In a previous publication a comparison of the physical properties of three pairs of epimeric acids was recorded. Two pairs were prepared synthetically from the corresponding pentoses and there was no doubt as to their configuration save the allocation of the amino groups. In the third pair, the configuration of one had been established by Fischer. Its epimer has been recently prepared from chitosaminic acid by the action of pyridine.<sup>1</sup> Since there was no precedent as to the reaction of pyridine on hexosaminic acid, the purity of what was expected to be epichitosaminic acid had to be established experimentally. The fact that epichitosaminic acid formed a crystalline lactone under exactly the same conditions which bring about the formation of a benzal ester of chitosaminic acid, and further, the fact that the lactone was converted into chitonic acid, seemed sufficient evidence in support of the assumed structure of epichitosaminic acid. Yet, additional proof seemed desirable, and because of this it was undertaken to convert the lactone into the corresponding sugar, with a view of re-converting the sugar into the  $\alpha$ -aminohexosaminic acid.

Another object in preparing the sugar was the following. On action of nitrous acid on chitosamine and the corresponding amino-

<sup>1</sup> Levene, P. A., *J. Biol. Chem.*, 1918, xxxvi, 73.



hexonic acid, and on chondrosamine and its acid, in each instance, the sugar and the acid gave rise to epimeric anhydro acids. On the action of nitrous acid on dextroxylohexosaminic acid and on its lactone also two epimeric anhydro acids were obtained. Since lactones may be regarded as inner esters, and since Fischer has observed that amino-acids and the corresponding esters as a rule gave antipodal hydroxy acid, the observation on xylohexosaminic acid was in harmony with what was to be expected.

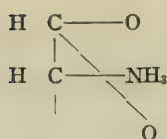
On the other hand, epichitosaminic acid and its lactone both gave, by the action of nitrous acid, chitonic acid. Instances where an amino-acid and its ester gave on deamination one and the same hydroxy acids were observed by Fischer. At first this author was inclined to assume that in such instances the Walden inversion did not occur; later he reconsidered his hypothesis and assumed that in these instances Walden inversion took place in both acid and ester. Both assumptions were more or less arbitrary and further experimental facts were desired for the correct interpretation of such observations. Because of these considerations it seemed desirable to test the action of nitrous acid not only on the lactone of epichitosaminic acid, but also on epichitosamine.

All attempts to carry out the original plan of the work proved futile. The sugar seemed to possess properties different from those observed on any other of the nitrogenous sugars.

First, the attempt to oxidize the sugar into the corresponding amino-acid failed. Both methods were employed; the one by means of bromine and the one by means of mercuric oxide. The temperatures and the duration of oxidation were varied, but the results were always negative.

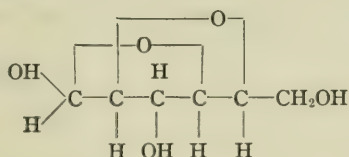
A second peculiarity of the sugar was its optical activity. It is the only sugar which showed no mutarotation. The substance was dissolved at 0°C. and the first reading of the solution taken immediately, but after 24 and after 48 hours no change in the initial reading was obtained. It seemed possible to explain this peculiarity by the assumption that this sugar in distinction from the others had the betain structure assumed by Irvine<sup>2</sup> for other amino sugars.

<sup>2</sup> Irvine, J. C., and Hynd, A., *J. Chem. Soc.*, 1912, ci, 1136.



If such a distinction between the individual sugars were a reality one should have expected that they would react differently towards nitrous acid. However, it was found that all known amino sugars gave off their nitrogen on treatment with nitrous acid in about 5 minutes.

The third peculiarity of the sugar lay in its behavior towards mercuric oxide. While other sugars under this treatment were transformed into the corresponding amino-acids, epiglucosamine formed under these conditions epichitose. The only pleasant surprise in the work on the sugars was the fact that epichitose, unlike chitose, appeared in beautiful crystalline form. Similar to epichitosamine, epichitose showed no mutarotation. Epichitose may be represented graphically as follows:



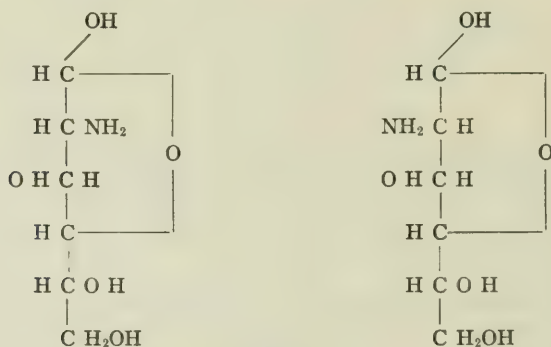
A fourth peculiarity of the sugar was found in its behavior towards nitrous acid. Previous to this, six hexosaminic acids, two lactones, and two hexosamines have been acted upon by nitrous acid. In every instance the resulting substance was a ring compound, owing to the dehydration following the deamination. On treatment of epichitosamine with nitrous acid, saccharic acid was obtained instead of anhydrosaccharic acid, as one would have expected. The saccharic acid was identified by the rotation of its acid potassium salt. The acid potassium salt of anhydrosaccharic acid crystallizes with one molecule of water of crystallization, and because of this the two salts are not analytically distinguishable. Fortunately they differ in their optical rotation. Also, the optical rotation of the calcium salt obtained on oxidation of epichitosamine showed the rotation of calcium salt

of saccharic acid. However, the calcium salt was obtained in small quantities and not analytically pure, but the evidence of the acid potassium salt is sufficient to establish the identity of the substance.

The fact that on deamination of the sugar saccharic acid was formed, whereas on the same treatment of the epichitosaminic acid and its lactone the anhydromannonic acid was formed is very important in connection with that phase of the discussion on Walden inversion, which has been referred to earlier in this paper; namely, when an amino-acid and the corresponding ester give rise to the same hydroxy acid then both theories are admissible, either that the inversion does take place in both of them, or in neither of them, and that the choice between the two for the present is arbitrary.

As regards the structure of the epichitosamine, mention must be made of the evidence in favor of its being an  $\alpha$ -amino sugar. It was found that the sugar on treatment with phenylhydrazine in the usual way gives rise to glucosazone, and on deamination of the lactone of epichitosaminic acid,  $\alpha$ - $\alpha_1$ -anhydromannonic acid is formed.

The graphic representation of the structure of epichitosamine can therefore be represented in one of the following ways.



#### EXPERIMENTAL.

The reduction of the lactone into the sugar was carried out in the same manner as described in previous communications. Lots of 10.0 gm. of lactone hydrochloride were taken up in 60.0 cc. of water and reduced with 125.0 gm. of a 2 per cent sodium amalgam. The

reaction of the solution was kept on the acid side by adding at short intervals small quantities of hydrochloric acid.

The filtrate from the mercury is concentrated at diminished pressure and at room temperature. Care has to be taken to carry out the concentration in such a manner that the concentrate remains colorless. The sodium chloride is separated by fractional precipitation first with methyl alcohol and subsequently with ethyl alcohol. After the product was obtained in such a state of purity that on ignition no ash was visible, the sugar was recrystallized once or twice out of water.

The analysis of the hydrochloride gave the following results. 0.1042 gm. of the substance gave 0.1298 gm. of  $\text{CO}_2$  and 0.0614 gm. of  $\text{H}_2\text{O}$ . 0.010 gm. of the substance gave in Van Slyke's micro-apparatus 1.18 cc. of nitrogen gas at  $T^\circ = 21^\circ\text{C}$ . and  $P = 765$ .

0.020 gm. of the substance gave in Van Slyke's micro-apparatus in 5 minutes 2.43 cc. of nitrogen gas at  $T^\circ = 25^\circ$  and  $P = 764$ .

The melting point of the substance was, M. P. =  $187^\circ\text{C}$ . (corrected).

	Calculated for $\text{C}_6\text{H}_{13}\text{NO}_3\text{HCl}$ . per cent	I-328 $\frac{1}{2}$ per cent	Found. II-575 $\frac{1}{2}$ per cent	III-111 $\frac{1}{2}$ per cent
C.....	33.40		33.97	
H.....	6.54		6.61	
N.....	6.51	6.73		6.78
Cl.....	16.45			

The rotation of the substance in 5 per cent HCl solution was

$$[\alpha]_D^{20} = \frac{\text{Initial.}}{1 \times 10} = -4.7 \quad [\alpha]_D^{20} = \frac{\text{Equilibrium.}}{1 \times 10} = -4.7$$

### *Preparation of the Osazone.*

4.6 gm. of the sugar hydrochloride were taken up in 200 cc. of water, the solution was neutralized with sodium acetate, and a solution of 8.0 gm. of phenylhydrazine in 5 cc. of glacial acetic acid were added and the entire solution was allowed to stand on the boiling water bath for 1 hour. An osazone formed which was recrystallized from methyl alcohol. M. P. at rapid heating was  $205^\circ\text{C}$ . It had the following composition. 0.100 gm. of the substance gave 0.2202 gm. of  $\text{CO}_2$  and 0.580 gm. of  $\text{H}_2\text{O}$ .



	Calculated for $C_{15}H_{22}N_4O_4$ per cent	Found. per cent
C. ....	60.33	60.05
H. ....	6.14	6.49

The rotation of the substance was, when 0.100 gm. was dissolved in 10 cc. of Neuberg's Pyridin and alcohol mixture: Initial — 0.31, Equilibrium — 0.15 which is in accord with the rotation found for glucosazone.

*Oxidation of Epichitosamine with Nitric Acid.*

Efforts to obtain the monocarboxylic acid failed, hence it was attempted to oxidize the sugar to the corresponding dicarboxylic acid. The conditions which led to a successful preparation of the substance are as follows. 10.0 gm. of the hydrochloride were dissolved in 100 cc. of water and to the solution were added 15.0 gm. of sodium nitrite and a few drops of concentrated hydrochloric acid. The flask was placed in an ice water bath and allowed to stand for 6 hours. At the end of that time the reaction product was placed on a boiling water bath for 3 minutes, filtered, and from the filtrate the excess of silver removed by means of hydrogen sulfide. The filtrate was concentrated to a volume of 35 cc. To this solution 35 cc. of concentrated nitric acid were added and the solution was rapidly evaporated on two large clock glasses. The residue was then dissolved by adding 5 cc. of water to each clock glass and again rapidly evaporated to dryness.

The residue was finally dissolved and converted into the calcium salt in the usual way. This salt did not analyze correctly for the expected dicarboxylic acid. However, the optical rotation of the substance differed from either anhydrosaccharic or anhydromannosaccharic, but closely approached that of saccharic acid.

The composition of the substance (No. 303<sup>18</sup><sub>19</sub>) was the following. 0.1042 gm. of the substance gave 0.1070 gm. of CO<sub>2</sub>, 0.0253 gm. of H<sub>2</sub>O, and 0.0270 gm. of CaO.

	Calculated for $C_6H_8O_6Ca$ per cent	Found. per cent
C. ....	29.03	28.00
H. ....	3.28	2.53
Ca. ....	22.58	25.91

The rotation was

$$\begin{array}{cc} \text{Initial.} & \text{Equilibrium.} \\ [\alpha]_D^{25} = \frac{+0.10 \times 100}{1 \times 2} = +5.0 & [\alpha]_D^{25} = \frac{+0.20 \times 100}{1 \times 2} = +10.0 \end{array}$$

\*Calcium salt of:

Anhydromannosaccharic.

$$\begin{array}{cc} \text{Initial.} & \text{Equilibrium.} \\ [\alpha]_D^{25} = \frac{+0.56 \times 100}{1 \times 2} = +28.0 & [\alpha]_D^{25} = \frac{+0.55 \times 100}{1 \times 2} = 27.5 \end{array}$$

Anhydrosaccharic.

$$[\alpha]_D^{25} = \frac{+0.55 \times 100}{1 \times 2} = +27.5 \quad [\alpha]_D^{25} = \frac{+0.58 \times 100}{1 \times 2} = 29.0$$

Saccharic acid.

$$[\alpha]_D^{25} = \frac{+0.09 \times 100}{1 \times 2} = +4.5 \quad [\alpha]_D^{25} = \frac{+0.22 \times 100}{1 \times 2} = 11.0$$

It was then concluded to prepare the acid potassium salt. For that purpose 20.0 gm. of epichitosamine chlorohydrate were oxidized in two lots of 10.0 gm. each in exactly the same manner as in the previous experiment. The product of oxidation of each lot was taken up in 8 cc. of water, the solution was rendered alkaline by means of a 50 per cent solution of potassium hydroxide, and then rendered acid by means of acetic acid. Methyl alcohol was added dropwise until slightly opalescent. The solution was then allowed to stand over night in the refrigerator. During that time a crystalline sediment formed. The sediment was recrystallized once out of dilute alcohol, and once out of water containing a few drops of acetic acid. The yield of the crude product was 1.9 gm.

The substance had the following composition. 0.1000 gm. of the substance gave 0.0352 gm. of  $K_2SO_4$ .

	Calculated for $C_6H_5O_5K$ . per cent	Found. per cent
K.....	15.70	15.81

The rotation of the substance in a 5 per cent solution of KOH was

$$[\alpha]_D^{25} = \frac{0.10 \times 100}{1 \times 2} = +5.0$$

Under the same condition a sample of the acid potassium salt obtained on oxidation of glucose possessed the following rotation.

$$[\alpha]_D^{25} = \frac{0.10 \times 100}{1 \times 2} = +5.0$$

A sample of the K salt of anhydrosaccharic acid showed the following rotation.

$$[\alpha]_D^{25} = \frac{+1.35 \times 100}{1 \times 2} = +67.5$$

Hence it is evident that the substance was the acid potassium salt of saccharic acid.

### *Epichitose.*

15.0 gm. of ash-free epichitosamine hydrochloride were taken up in 250 cc. of water and 80.0 gm. of mercuric oxide, and heated on a boiling water bath for 30 minutes at the end of which time the greater part of oxide turned from orange to gray. Through the filtrate from the oxide hydrogen sulfide gas was passed and the filtrate from the sulfide was concentrated to a very thick syrup. This was taken up with a little methyl alcohol. On standing over night a crystalline deposit formed. It was nitrogen-free, it reduced Fehling's solution on heating, and possessed a melting point, M. P. = 240° (corrected), with decomposition.

The analysis of the substance gave the following results. 0.1031 gm. gave 0.1670 gm. of CO<sub>2</sub> and 0.0570 gm. of H<sub>2</sub>O.

	Calculated. per cent	Found, per cent
C .....	44.44	44.18
H .....	6.17	6.18

The optical rotation of the substance was

$$[\alpha]_D^{25} = \frac{\text{Initial.}}{1 \times 2} = \frac{-1.92 \times 100}{1 \times 2} = -96.0 \quad [\alpha]_D^{25} = \frac{\text{Equilibrium.}}{1 \times 2} = \frac{-1.92 \times 100}{1 \times 2} = -96.0.$$

## CYTIDINE PHOSPHORIC ACID.

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The theoretical importance attached to the nucleotides obtained on acid hydrolysis of yeast nucleic acid has been discussed in a previous publication. It was there pointed out that the product of the acid hydrolysis was a mixture of two mononucleotides;<sup>1</sup> namely, of uridine phosphoric and of cytidine phosphoric acids. The former had been obtained in a crystalline form as a barium salt, the latter was prepared also as a barium salt. The barium salt of cytidine phosphoric acid possessed greater solubility in water, and had a tendency to settle out of a very concentrated solution in the form of a granular precipitate which consists of microscopic granules. On recrystallization it formed a deposit of irregular plates. The precipitate of barium uridine phosphate, when it settles out rapidly from its aqueous solution, not infrequently has a similar appearance. The difference in the behavior of the two salts is that barium uridine phosphate on recrystallization settles out in form of prismatic needles while the salt of cytidine phosphoric acid on recrystallization appears in form of microscopic granules.

### EXPERIMENTAL.

The conditions of hydrolysis and of preparation of the crude brucine salts were given in the communication on the uridine phosphoric acid. The purification of the brucine salt of cytidine phosphoric acid was perfected since the date of the older publication. The process as carried out at present is as follows.

The mixed brucine salt is dissolved in boiling 35 per cent alcohol in the proportion of 15.0 liters of alcohol to 100.0 gm. of the salts. The solution is allowed to cool and is kept at 10°C. for 24 hours. A

<sup>1</sup> Levene, P. A., *Proc. Soc. Exp. Biol. and Med.*, 1917, xv, 21; *J. Biol. Chem.*, 1918, xxxiii, 229.



crystalline deposit of brucine salts is formed, which is separated by filtration and again dissolved in 35 per cent alcohol in the same proportions as above. The process is repeated once more. The mother liquors of the three crystallizations are combined and concentrated under diminished pressure to a small volume. A crystalline deposit forms in the distilling flask. To complete the crystallization the contents of the flasks are allowed to stand over night at  $10^{\circ}\text{C}$ .

This product consists chiefly of the brucine salt of cytidine phosphoric acid, but contains a small amount of uridine phosphoric acid. The greater part of the latter is removed in the following way. The material is taken up in methyl alcohol in the proportion of 25.0 gm. of the brucine salts to 1 liter of alcohol and the alcohol is brought to a boil on the water bath. The greater part of the brucine salt of uridine phosphoric acid remains insoluble. The extract contains the salt of cytidine phosphoric acid. A part of it settles out on cooling the solution; the remainder crystallizes after the mother liquor is concentrated under diminished pressure to small volume.

The following analytical data of the three fractions represent the results of one of many similar experiments.

I.—Part insoluble in hot methyl alcohol.

0.200 gm. gave 13.8 cc. of N at  $T^{\circ} = 30^{\circ}\text{C}$ .  $P = 757$  mm.  $N = 7.74$  per cent.

II.—Part crystallized on cooling of hot extract.

0.200 gm. gave 15.6 cc. of N at  $T^{\circ} = 30^{\circ}\text{C}$ .  $P = 757$  mm.  $N = 8.75$  per cent.

III.—Part crystallized on concentration of mother liquor.

0.200 gm. gave 14.4 cc. of N at  $T^{\circ} = 14^{\circ}\text{C}$ .  $P = 759$  mm.  $N = 8.27$  per cent.

The theory for the brucine salt of uridine phosphoric acid  $\text{C}_{55}\text{H}_{63}\text{N}_6\text{PO}_7 + 7\text{H}_2\text{O}$  requires  $N = 6.79$  per cent.

The theory for the brucine salt of cytidine phosphoric acid  $\text{C}_{63}\text{H}_{64}\text{N}_7\text{PO}_6 + 7\text{H}_2\text{O}$  requires  $N = 7.93$  per cent.

#### *Conversion of the Brucine Salts into Barium Salts.*

The old process of this conversion has been modified, since it was found to be associated with considerable loss. The brucine salts are dissolved in boiling 35 per cent alcohol, to the solution an excess of ammonia is added, and the ammoniacal solution is concentrated under diminished pressure until free brucine begins to crystallize.

The brucine is removed by filtration, the mother liquor is again rendered strongly ammoniacal, and concentrated until brucine begins to crystallize. The operation was repeated as often as required, so that finally on concentration no perceptible quantity of brucine became evident. The brucine which separates out on concentration of the ammoniacal solution contains some of the brucine salts of nucleotides, and it is advisable to suspend them in hot water, treat the suspension with an excess of ammonia, filter them from free brucine, and to concentrate the mother liquor as above.

The next step in the process consists in obtaining the ammonium salts of the nucleotides. For this purpose they are precipitated out of their concentrated solution by absolute alcohol, by acetone, or by a mixture of equal parts of acetone and alcohol. The choice of the reagent depends upon the solubility of a given nucleotide, and if the solubility is not known a test should be made on a small sample of the solution of the ammonium salts. Thus the ammonium salt of uridine phosphoric acid is precipitated readily by means of absolute alcohol. The salt of cytidine phosphoric acid is precipitated more conveniently by a mixture of equal parts of alcohol and acetone. The yield of ammonium salts from the brucine salts is practically theoretical.

For conversion into the barium salt the ammonium salt of the nucleotide is dissolved in water and a slight excess of the theoretical amount of aqueous solution barium hydroxide is added. The solution is then evaporated at room temperature under diminished pressure nearly to dryness. This operation is repeated three times. The final residue is dissolved in a slight excess of dilute sulfuric acid bringing the volume to 1 liter for every 20 gm. of the ammonium salt. To this solution aqueous barium hydroxide is added until the solution turns very faintly pink with phenolphthalein. The product is then filtered and the filtrate is concentrated under diminished pressure and at room temperature until the solution in the distilling flask turns slightly turbid. The contents of the flask are then allowed to stand at room temperature over night, filtered, and the filtrate is again concentrated as the first time. The operation is repeated as often as necessary.

The barium salts may be recrystallized by dissolving in water containing a slight excess of sulfuric acid. The solution is then treated in exactly the same manner as for the first precipitation of the barium salts.

The analysis of the barium salt of cytidine phosphoric acid gave the following results.

0.0842 gm. of substance used for Kjeldahl estimation required for neutralization 5.65 cc. of 0.1 N acid.

0.1316 gm. of substance gave 0.1144 gm. of  $\text{CO}_2$ , 0.0300 gm. of  $\text{H}_2\text{O}$ , and 0.0642 gm. of  $\text{Ba}_2\text{P}_2\text{O}_7$ .

0.1684 gm. of the substance gave 0.0388 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

	Calculated for $\text{C}_8\text{H}_{12}\text{N}_3\text{O}_8\text{PBa}$ , per cent	Found, per cent
C.....	23.56	23.71
H.....	2.64	2.55
N.....	9.16	9.39
P.....	6.77	6.43
Ba.....	29.96	29.36
$\text{Ba}_2\text{P}_2\text{O}_7$ .....	48.97	48.79

The optical rotation of the air-dry substance was

$$[\alpha]_D^{20} = \frac{+0.56 \times 100}{1 \times 4} = +14.0$$

#### *Hydrolysis of the Substance.*

8.0 gm. of the barium salt were suspended in 100 cc. of a 10 per cent solution of sulfuric acid and heated for 4 hours in a sealed tube at  $125^\circ$  in an oil bath. The product was filtered from barium sulfate. From the filtrate the phosphoric and sulfuric acids were removed by an excess of barium hydroxide and the excess of barium hydroxide removed quantitatively. The filtrate was then concentrated to a small volume and treated with an aqueous solution of picric acid. A light yellow precipitate formed which consisted of microscopical striated droplets. The mother liquor on further concentration gave a second and then a third deposit, all of identical appearance. The combined picrates were washed with ether until the washings became practically colorless. The substance was recrystallized out of 95 per cent alcohol three times. The melting point of the substance was

the following: at 140°C. (not corrected) it became transparent, at about 180°C. it began to darken, and at 200°C. it decomposed with the evolution of gas. A sample of cytidine picrate of our collection which was recrystallized in the same manner behaved identically.

For measuring the optical rotation the picrate was dissolved in a warm solution of 1.0 per cent hydrochloric acid, filtered from picric acid which separated on cooling, and the filtrate was used for the measurement. The value found was

$$[\alpha]_D^{20} = \frac{+0.19 \times 100}{1 \times 1} = +19.0$$

A sample of cytidine picrate previously prepared gave the value

$$[\alpha]_D^{20} = \frac{+0.20 \times 100}{1 \times 1} = +20.0$$

The analysis of the substance gave the following results.

0.1122 gm. of the substance gave 17.4 cc. of nitrogen at  $T^\circ = 21^\circ\text{C.}$  and  $P = 760$ .  
0.09928 gm. of the substance gave 0.1392 gm. of  $\text{CO}_2$  and 0.0322 gm. of  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_8\text{H}_{13}\text{O}_5\text{N}_3 \cdot \text{C}_6\text{H}_5(\text{NO}_2)_3\text{OH}$ <i>per cent</i>	Found. <i>per cent</i>
C.....	38.14	37.94
H.....	3.40	3.57
N.....	17.79	17.74

Thus the product of hydrolysis was cytidine where cytosine was expected. No explanation can be given for the unexpected result. Possibly the sulfuric acid solution employed in the experiment contained less than 10 per cent of sulfuric acid. However, for the proof of the nature of the nucleotide the isolation of either cytosine or cytidine has the same value. No uridine was found in the mother liquors from the picrate. In fact on evaporation to dryness and extraction with ether the residue was insignificant.





## LIPOIDS OF THE HEART MUSCLE.

BY P. A. LEVENE AND S. KOMATSU.

*(From the Laboratories of The Rockefeller Institute for Medical Research.)*

(Received for publication, June 30, 1919.)

### INTRODUCTORY REMARKS.

BY P. A. LEVENE.

The work on the phosphatides of the heart muscle has made us see the old distinctions between individual phosphatides in a novel light. Prior to this work most workers distinguished phosphatides first by their solubility, and second by the ratio of the elements of nitrogen and phosphorus.

By their solubility all unsaturated phosphatides were generally classified into three fractions: (1) lecithin, the fraction soluble in ether and alcohol; (2) cephalin, the fraction soluble in ether and insoluble in alcohol, hence obtained by precipitating the ethereal solution with alcohol; and (3) acetone-soluble fraction.

The lecithin group was regarded as consisting practically entirely of lecithin.

The cephalin fraction of individual organs was claimed to consist of substances varying in their composition with the variation of the organ.

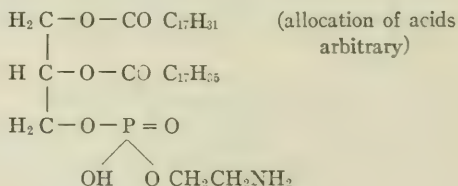
The same applied to the acetone-soluble fraction.

Through the work done in this laboratory evidence was furnished in favor of the view that both the lecithin and acetone-soluble fractions are composed of a mixture in varying proportions of lecithin and cephalin, each respectively having the structure as required by theory. The mixture can be separated into its component parts by reducing the crude material with hydrogen gas in the presence of colloidal palladium, and subsequently separating hydrocephalin from hydrolecithin by fractional precipitation out of organic solvents.

On the other hand, the so called cephalin fraction was proved to consist of a mixture of fragments (of various size) of both lecithin and of cephalin. True, in the cephalin fraction the cephalin fragments predominate over those of lecithin, and *vice versa*, in the lecithin fraction the predominating part is unchanged lecithin. But it is also true that the generally accepted view on the two fractions "lecithin" and "cephalin" has to be radically changed, and that part of the ether-soluble material which is also soluble in alcohol should be regarded as the fraction containing the unaltered lecithin and cephalin, and the fraction which is soluble in ether and insoluble in alcohol as consisting principally of mixtures of fragments of lecithin and cephalin. This contention has been proved definitely for the lipoids of the heart muscle. There is reason to believe that the same applies to all other organs. The correctness of this contention is being further tested in this laboratory.

### *I. Hydrolecithin and Hydrocephalin of Heart Muscle.*

In previous communications by Levene and West<sup>1,2</sup> on the phosphatides of the egg yolk, it was shown that the so called lecithin was a mixture of lecithin and cephalin. When reduced by means of hydrogen in the presence of colloidal palladium the material was fractionated into hydrocephalin and hydrolecithin. Through that observation proof was furnished in support of the generally assumed structural formula of cephalin, as follows:



The analysis of the so called lecithin fraction obtained from the heart muscle also consists of a mixture of the same two components. When the lecithin fraction was reduced by means of hydrogen in

<sup>1</sup> Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1918, xxxv, 285.

<sup>2</sup> Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1918, xxxiv, 175.

the presence of colloidal palladium a product was obtained which showed a nitrogen distribution  $\frac{\text{NH}_2\text{N}}{\text{Total N}} = \frac{31}{100}$ , thus containing nearly one-third of cephalin. The material had all the properties of the crude hydrolecithin obtained from egg yolk. By the process which had been applied for fractionation of the latter material the crude hydrolecithin of the heart muscle was fractionated into two fractions, one consisting principally of hydrocephalin (77 per cent), and the other principally of hydrolecithin (76 per cent). Further purification to substances of greater purity is only a question of material and work.

The composition of the respective samples was as follows:

	Hydrolecithin. $\text{C}_{44}\text{H}_{90}\text{O}_8\text{NP}$		Hydrocephalin. $\text{C}_{41}\text{H}_{82}\text{O}_8\text{NP}$	
	Calculated. <i>per cent</i>	Found. <i>per cent</i>	Calculated. <i>per cent</i>	Found. <i>per cent</i>
C.....	65.37	65.86	65.80	65.50
H.....	11.23	11.39	11.05	10.36
N.....	1.74	1.86	1.87	1.80
P.....	3.84	4.06	4.15	4.29
$\frac{\text{NH}_2\text{N}}{\text{Total N}}$	$\frac{0}{100}$	$\frac{35}{100}$	$\frac{100}{100}$	$\frac{77}{100}$

On repeated recrystallization:

$\frac{\text{NH}_2\text{N}}{\text{Total N}}$	$\frac{24}{100}$
$[\alpha]_D^{20}$	+ 4.0                      + 3.3

These observations contribute to the evidence in favor of the structural formula of cephalin. The observations are important also from the view-point of another more general consideration. An opinion has been expressed with particular emphasis by Fränkel and Linnert<sup>3</sup> that the individual organs of the same animal contain specific phosphatides and further, that identical organs of animals of different species also contained distinct phosphatides. The experience gained in this laboratory, however, has been that as the methods of isolation

<sup>3</sup> Fränkel, S., and Linnert, K., *Biochem. Z.*, 1910, xxiv, 270.



of the individual lipoids are being perfected and as the knowledge of the structure of individual substance is making progress, so the conviction grows that the number of individual lipoids is rather limited, and that practically all animal organs contain the same lipoids. This view has been expressed by one of us on several other occasions.

#### EXPERIMENTAL.

160.0 gm. of lecithin with a ratio of  $\frac{\text{NH}_2\text{N}}{\text{Total N}} = \frac{32}{100}$  were reduced in the same manner as described by Levene and West. The yield of reduced material after one reduction was 70.0 gm. These were dissolved in hot 95 per cent alcohol and allowed to stand at 10° for 24 hours. 44.0 gm. of the substance settled out on cooling. This material had a ratio of  $\frac{\text{NH}_2\text{N}}{\text{Total N}} = \frac{31}{100}$ . This material was recrystallized out of a solvent consisting of equal parts of chloroform and methylethyl ketone. 15 parts of the solvent were used for 1 part of the material. The process was repeated once. The final yield was 25.0 gm. This material was then dissolved in hot chloroform and the solution poured into three volumes of 99.5 per cent alcohol. The precipitate was filtered immediately; the yield was 5.0 gm.

The substance obtained in this manner had the following composition:

0.0992 gm. of substance gave 0.2336 gm. of CO<sub>2</sub>, 0.0910 gm. of H<sub>2</sub>O, and 0.0115 gm. of ash.

0.127 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 1.60 cc. of 0.1 N acid.

0.1622 gm. of substance gave on fusion 0.0245 gm. of Mg<sub>2</sub>P<sub>2</sub>O<sub>7</sub>.

2 gm. were hydrolyzed and made up to 20 cc. 5 cc. of the solution required 1.05 cc. of 0.1 N acid; 2 cc. of the solution gave 0.81 cc. of N at 26°, *P* = 760.7 mm.

The rotation of the substance in chloroform was

$$[\alpha]_D^{25} = \frac{+0.10 \times 100}{1 \times 3} = +3.3$$

Since this rotation differed numerically from that made on the hydrocephalin and since the measurements in the present work were made under slightly different conditions from those of the previous

year, the measurement of the rotation of the hydrolecithin from the egg yolk was repeated under the present conditions.

The result was as follows:

$$[\alpha]_D^{25} = \frac{+0.11 \times 100}{1 \times 3} = +3.6$$

The mother liquors resulting from crystallization of the material out of solution of chloroform and methylethyl ketone were concentrated and recrystallized out of alcohol. The yield was 10.0 gm. The substance had the following composition:

0.0988 gm. of the substance gave 0.2388 gm. of  $\text{CO}_2$ , 0.1006 gm. of  $\text{H}_2\text{O}$ , and 0.0101 gm. of ash.

0.1868 gm. of the substance used for Kjeldahl nitrogen estimation required for neutralization 2.45 cc. of 0.1 N acid.

0.1586 gm. of the substance gave on fusion 0.0228 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

After repeated recrystallization the ratio of  $\frac{\text{NH}_2\text{N}}{\text{Total N}} = \frac{24}{100}$ .

2 gm. of the substance were hydrolyzed and made up to 25 cc.

5 cc. of the solution required 2.15 cc. of 0.1 N acid.

2 cc. of the solution gave 0.56 cc. of N at  $31^\circ$ .  $P = 756$  mm.

$$\frac{\text{NH}_2\text{N}}{\text{Total N}} = \frac{24}{100}$$

The rotation of the substance in chloroform was

$$[\alpha]_D^{25} = \frac{0.12 \times 100}{1 \times 3} = +4.0.$$

## II. Acetone-Soluble Phosphatides.

It was shown by several observers that the acetone ethereal mother liquors, resulting from precipitation of an ethereal solution of lecithin by acetone, contained phosphatides. Fränkel and his pupils claimed to have discovered among them a variety of new phosphatides. MacLean on the contrary expressed the view that these phosphatides consisted in the main of lecithin.<sup>4</sup> The conclusions of MacLean seemed more acceptable to us particularly in the light of the observation of Levene and West on the phosphatide obtained from egg yolk.<sup>2</sup>

<sup>4</sup> MacLean, H., *Biochem. J.*, 1914, viii, 453.

Also in the acetone residue from the heart muscle the phosphatides consisted principally of lecithin and secondarily of a small mixture of cephalin. The process of preparation was slightly modified from that described by Levene and West.

The lipoids were prepared in the form of the cadmium chloride salt. The ratio  $\frac{\text{NH}_2\text{N}}{\text{Total N}} = \frac{1}{100}$ .

The optical rotation of the cadmium salt was:  $[\alpha]_D^{25} = +4.4$ .

#### EXPERIMENTAL.

The acetone-ether solutions were concentrated under reduced pressure to a thick syrup. This was slightly diluted with alcohol and an alcoholic solution of cadmium chloride was added as long as a precipitate formed. The precipitate which had at first a very sticky character was extracted with acetone five times until it settled as a fine dry powder. This substance had the following composition:

0.1757 gm. of the substance employed for Kjeldahl nitrogen estimation required 1.95 cc. of 0.1 N acid for neutralization; N = 1.56 per cent.

0.2548 gm. of the substance gave on fusion 0.0270 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ ; P = 2.98 per cent. The ratio of  $\frac{\text{NH}_2\text{N}}{\text{Total N}} = \frac{7}{100}$ .

This was suspended in ether and water was added gradually in very small portions until the solution of the salt was completed. The solution was then poured into alcohol. This operation was repeated several times.

The final product had the following composition:

0.100 gm. of the substance gave on combustion 0.1671 gm. of  $\text{CO}_2$ , 0.0634 gm. of  $\text{H}_2\text{O}$ , and 0.0258 gm. of ash.

0.251 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 2.75 cc. of 0.1 N acid.

0.1700 gm. of the substance gave on fusion 0.0195 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

0.500 gm. of the substance gave 0.1506 gm. of  $\text{CdSO}_4$ .

2.0 gm. of the substance were hydrolyzed with hydrochloric acid as usual. The solution was neutralized and made up to 20.0 cc.

5 cc. of this solution required for neutralization 4.25 cc. of 0.1 N acid.

2 cc. of the solution gave 0.06 nitrogen gas at  $T^\circ = 29^\circ\text{C}$ . and  $P = 759$  mm.

	Calculated for $C_{44}H_{86}O_9N_2P_2Cd_2Cl_4$ .	Found.
	<i>per cent</i>	<i>per cent</i>
C.....	45.13	45.57
H.....	7.35	7.09
N.....	1.19	1.53
P.....	2.65	3.09
Cd.....	19.20	16.21
$NH_2N$	0	1
Total N	100	100

Thus the substance consisted practically of the dicadmium salt, containing perhaps an insignificant proportion of the monocadmium derivative.

The optical rotation of the substance was

$$[\alpha]_D^{25} = \frac{+0.11 \times 100}{0.5 \times 5} = +4.4$$





## CEPHALIN.

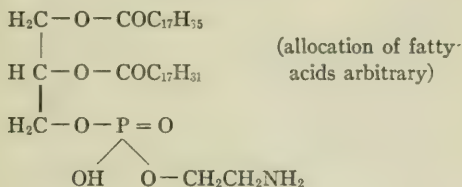
### VI. THE BEARING OF CUORIN ON THE STRUCTURE OF CEPHALIN.

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(Received for publication, June 30, 1919.)

The hitherto isolated components of cephalin are phosphoric acid, glycerol, aminoethanol, and stearic and linoleic acids. A molecule composed of equimolecular proportions of these substances may be represented by the following graphic formula.



This molecule requires the following percentage composition of the elements entering into it.

	C	H	N	P
Per cent.....	66.16	10.57	1.88	4.18

The values generally found on analysis by various investigations were

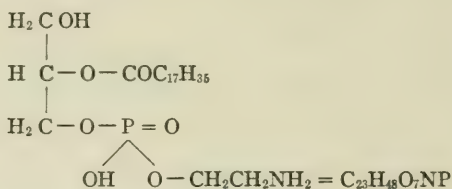
	C	H	N	P
Per cent.....	60.13	9.50	1.80	3.70

This discrepancy between theory and fact never received a satisfactory explanation.

*A priori* three alternative explanations were possible: first, cephalin possessed a structure different from the one expressed by the given formula; second, the cephalin isolated by the usual methods represented a substance modified in the process of preparation; and third, the substance was a mixture of true cephalin with some other substance of unknown composition.

Recently Levene and West<sup>1</sup> have prepared a reduced cephalin which possessed an elementary composition required by the structural formula given above. This find excludes the first of the three alternatives, and the choice remains between the second and the third.

The present communication offers an explanation to the puzzling analytical data obtained on cephalin by all workers beginning with Thudichum. In the light of the work to be reported here both the second and the third alternatives combine to bring about the known empirical composition of the so called cephalin. It appears that cephalin is a mixture of pure cephalin with decomposition products of it. One of the decomposition products is cephalin from which one fatty acid has been removed either by the chemical manipulations or by enzymes. A substance of this nature should have the following composition.



This substance should give the following analytical values.

	C	H	N	P
Per cent.....	57.3	9.9	2.7	6.4

A substance in its elementary composition approaching this hypothetical fragment of cephalin was isolated. It contained

	C	H	N	P
Per cent.....	56.93	9.23	2.14	6.05

Substances were obtained also which consisted apparently of products of deeper deterioration of the original cephalin molecule, thus a fraction was isolated which analyzed as follows

	C	H	N	P
Per cent.....	54.43	8.70	1.46	6.42

which was perhaps a mixture of the monostearyl-aminoethanol derivative of glycerophosphoric acid with stearyl-glycerophosphoric acid

<sup>1</sup> Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1918, xxxv, 285.

and perhaps with some glycerophosphoric acid. The theory for monostearyl-glycerophosphoric acid requires the following values.

Calculated for  $C_{21}H_{45}O_3NP$  (molecular weight 452)

	C	H	N	P
Per cent. ....	55.7	9.57	0	6.85

In other words the so called cephalin consists of true cephalin and of all the products of its intermediate hydrolysis; namely, of monostearyl-glycerophosphoric aminoethanol ester, monostearyl-glycerophosphoric acid, and glycerophosphoric acid. This conclusion was arrived at in the course of an investigation into the chemical nature of cuorin.

### *Cuorin.*

Cuorin was described by Erlandsen<sup>2</sup> as an individual phosphatide differing from both lecithin and cephalin. By the method of preparation and by its physical properties cuorin showed close resemblance to cephalin. The difference lay in the values of the elements nitrogen and phosphorus present in the molecules of the two substances. In cephalin the values are  $N = 1.88$ ,  $P = 4.18$ , and the ratio  $N : P = 1 : 1$ , whereas in cuorin the values are 1 and 4 respectively and the ratio  $N : P = 1 : 2$ .

However, there were reasons to suspect that cuorin was not an individual substance but a mixture of cephalin with other substances. Maclean,<sup>3</sup> who described substances resembling Erlandsen's cuorin, expressed, in his monograph on lecithin, doubts as to the existence of cuorin as an individual substance. These doubts are accentuated by the observations made in the course of the present work. First, by apparently identical methods of preparation substances were obtained with the composition approaching either that of cuorin or that of the so called cephalin. Second, from a substance with the composition of cuorin, depending on the mode of treatment could be obtained a large proportion of a substance resembling cephalin or *vice versa*. Third, as in crude cephalin or lecithin the basic com-

<sup>2</sup> Erlandsen, A., *Z. physiol. Chem.*, 1907, li, 71.

<sup>3</sup> Maclean, H., Lecithin and allied substances. The lipins, *Monographs on Biochemistry*, London, 1918, 52.



ponent of the substance was not uniform, but consisted of a mixture of choline and aminoethanol and on fractionation the individual fraction did not contain the bases in stoichiometric proportions. On the contrary, the fractions more soluble in alcohol contained progressively smaller proportions of aminoethanol with the increase of their solubility, and *vice versa*. Fourth, the proportion of fatty acids varied irregularly in various fractions.

The greatest part of cuorin, however, is made up of the material generally called crude cephalin. Thus every sample of cuorin, if purified by precipitation out of aqueous solution by hydrochloric acid, contains the elements nitrogen and phosphorus in ratio of  $N : P = 1 : 1$ . A sample of material was prepared according to the process of Erlandsen, and it had the composition:

	C	H	N	P
Per cent. ....	61.85	8.42	1.66	4.60

and the ratio  $N : P = 1 : 1.25$ . From this substance at will could be obtained either a substance analytically resembling cuorin or cephalin.

Thus when allowed to settle out on cooling out of hot methylethyl ketone, and subsequently allowed to settle out on cooling out of hot ethylacetate a material was obtained having the values of the elements of nitrogen and phosphorus 1.22 per cent and 4.15 per cent respectively, or the ratio  $N : P = 1 : 1.54$ . When the same material was purified by the hydrochloric acid process a substance was obtained which was separated into two fractions, one having  $N = 2.04$  and  $P = 4.07$ , the ratio  $N : P = 1 : 1$  approximately; and the other  $N = 1.61$  and  $P = 4.13$ , the ratio  $N : P = 1 : 1.17$ . In another experiment the material having  $N = 1.6$  and  $P = 4.4$  and  $N : P = 1 : 1.25$  was separated by means of methylethyl acetone into two fractions, one having  $N = 1.6$  and  $P = 4.0$ ,  $N : P = 1 : 1.13$ ; and the other having  $N = 1.86$  per cent and  $P = 8.40$  per cent,  $N : P = 1 : 2.03$ .

The basic components of the individual samples varied considerably.

Thus, the proportion of  $\frac{NH_2N}{\text{Total N}}$  varied from 0.70 to 0.45. Therefore,

it seemed clear that cuorin was a mixture. The question then arose as to the nature of the materials which make up the so called cuorin.

It was concluded that these were the same fragments of cephalin and lecithin which make up the so called crude cephalin. Some of the fragments were enumerated in the opening chapter of this communication.

*Reduction of the Cephalin Fraction of Cuorin.*

For convenience of the work on the nature of the material obtained from so called cuorin and resembling cephalin the material was reduced by means of hydrogen in the presence of colloidal palladium. It was found that the reduction proceeded more satisfactorily when the substance had been purified by the hydrochloric acid process. Just as in the earlier experience of Levene and West<sup>4</sup> it was also found that the reduction of cephalin proceeded at a very slow rate and that the volume of absorbed hydrogen was comparatively small.

The main product obtained after reduction had an elementary composition nearly identical with that obtained by different investigators on unreduced cephalin. The values obtained by Thudichum<sup>5</sup> and by Levene and West on unreduced cephalin were

	C	H	N	P
	per cent	per cent	per cent	per cent
Thudichum.....	60.00	9.38	1.68	4.27
Levene and West.....	60.33	9.62	1.78	3.60

and on reduced cephalin

Levene and Komatsu.....	59.47	9.41	1.68	4.17
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The material contained about 92 per cent of its nitrogen in the form of amino nitrogen. Thus it seemed as if some factor was interfering with the reduction of cephalin. Two alternative assumptions were capable of explaining this observation; first, that the unsaturated fatty acid through oxidation was converted into hydroxyacid which could not naturally be reduced by hydrogen in the presence of a catalyst; second, that the unsaturated fatty acid was prevented from absorbing hydrogen by the presence of an impurity acting as a catalytic poison.

<sup>4</sup> Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1916, xxiv, 45.

<sup>5</sup> Thudichum, J. L. W., *The chemical constitution of the brain*, London, 1884, 57.

Under the first assumption an hydroxyacid should be found among the hydrolytic products of the above material, under the second, unsaturated fatty acids. Neither one of the two expectations was realized. On hydrolysis of the material there was found only one fatty acid; namely, stearic acid. Furthermore, the value of the fatty acids in the material was found to be 64.16 per cent. A value of 63.40 per cent was previously found by Levene and West on unreduced cephalin. The theory for the cephalin molecule containing two molecules of fatty acids requires 75.90 per cent of the acids, and the theory of the fragment with one fatty acid requires 58.84 per cent. On the basis of these considerations it was assumed that the material was a mixture of cephalin with its fragments. Indeed on modifying the mode of treatment there were obtained fractions of varying composition.

	C <i>per cent</i>	H <i>per cent</i>	N <i>per cent</i>	P <i>per cent</i>
I.....	62.51	9.68	1.58	4.01
II.....	62.13	9.36	2.04	4.17
III.....	61.94	9.47	2.43	5.81
IV.....	61.03	10.11	2.00	5.15
V.....	56.93	9.23	2.14	6.05
VI.....	54.43	8.70	1.46	6.42

Of these (V) in composition approaches monostearyl-glycerophosphoric aminoethanol ester. Taking into consideration that every one of these fragments is composed of some or of all of the following radicles, that of phosphoric acid, of glycerol, of stearic acid, and of aminoethanol; taking further into consideration that, judging from the elementary analysis, the radicles are present in proportions that have no stoichiometric relationship, one is inclined to assume that every one of the fractions is a mixture of the fragments of cephalin, also that the so called cephalin (C = 60.0 per cent approximately) is a mixture of such fragments, and that cuorin differs from cephalin only inasmuch as it contains a larger proportion of lower fragments.

#### EXPERIMENTAL.

##### *Preparation of Crude Cuorin.*

Heart muscle was minced in meat chopping machines, dried in vacuum driers, and pulverized. The dry powder was extracted with ether containing 7.0 per cent water, since it was found that anhy-

rous ether extracted only minimal quantities of phosphatides. The ethereal solution was concentrated to small volume and to it dry acetone was added as long as a precipitate was formed. The precipitate was dried under diminished pressure over sulfuric acid, redissolved in ether, and allowed to stand at 0°C. over night to allow the "white matter" to settle out. The ethereal solution was again concentrated and treated with acetone. The operation was repeated many times as the new ethereal solution on standing at low temperature still formed a sediment of white matter. The final product obtained in this manner was further treated according to Erlandsen, and the product insoluble in 95 per cent alcohol at 60°C. was expected to be cuorin. However, the ratio of the elements nitrogen to phosphorus was found greater than the one required for cuorin and smaller than that for cephalin; namely,  $\frac{N}{P}$  was greater than  $\frac{1}{2}$  and smaller than

Under similar treatment Erlandsen obtained a cuorin having the elements nitrogen and phosphorus in the ratio  $\frac{N}{P} = \frac{1}{2}$ .

### *Analysis of Crude Cuorin.*

*Sample A.*—0.2519 mg. of the substance used for Kjeldahl nitrogen estimation required 2.9 cc. of 0.1 N acid for neutralization, N = 1.6 per cent.

0.2493 gm. of the substance on fusion gave 0.0395 gm. of  $Mg_2P_2O_7$ , P = 4.4. Ratio of N : P = 1 : 1.25.

2 gm. were used for the estimation of the distribution of nitrogen. The filtrate after hydrolysis was made up to 15 cc. 5 cc. used for total nitrogen estimation required for neutralization 1.2 cc. of 0.1 N acid. 2 cc. used for amino nitrogen estimation gave 0.85 cc. of nitrogen at  $T = 22^\circ$ ,  $P = 758.6$  mm.  $\frac{NH_2N}{Total\ N} = \frac{70}{100}$ .

*Sample B.*—The analysis of this sample gave the following results. 0.100 gm. of the dry material gave 0.2180 gm. of  $CO_2$ , 0.0724 gm. of  $H_2O$ , and 0.014 gm. of H.

0.2885 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 3.3 cc. of 0.1 N acid.

0.3012 gm. of the substance gave on fusion 0.0477 gm. of  $Mg_2P_2O_7$ .

$$\frac{NH_2N}{Total\ N} = \frac{65}{100}$$

	C	H	N	P
Hence ash-free per cent. . . . .	61.85	8.42	1.66	4.60



*Fractionation and Purification of Crude Cuorin.*

*The first step* in fractionation of the crude cuorin was accomplished by means of methylethyl ketone. The material dried under diminished pressure was dissolved in the boiling reagent. Under these conditions a small part remained insoluble. This fraction obtained from Sample A had a composition of N = 1.86 per cent and P = 8.40 per cent, from Sample B, N = 2.54 per cent and P = 5.74 per cent. The soluble part on cooling formed a precipitate. In Sample A this fraction had the following composition.

0.3452 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 3.9 cc. of 0.1 N acid.

0.2924 gm. of the substance gave on fusion 0.042 gm. of  $Mg_2P_2O_7$ .

$$N = 1.6 \text{ per cent and } P = 4.0 \text{ per cent; } \frac{N}{P} = \frac{1}{1.13}; \frac{NH_2N}{\text{Total N}} = \frac{70}{100}$$

*The second step* in the purification of the fraction soluble in hot methylethyl ketone was accomplished in the following two manners: (a) it was recrystallized out of dry ethylacetate; (b) it was taken up in water and placed in a shaking machine until the mixture acquired the character of an opalescent viscous liquid. From this the substance was precipitated by means of 10 per cent hydrochloric acid. The precipitate was separated either by filtration or by centrifugation. The sediment was washed with acetone, then dissolved in ether, and poured into alcohol. The material obtained by the first process contained the elements nitrogen and phosphorus in the following proportions.

0.3055 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 2.58 cc. of 0.1 N acid.

0.2479 gm. of the substance gave on fusion 0.0382 gm. of  $Mg_2P_2O_7$ .

$$N = 1.18 \text{ per cent and } P = 4.31 \text{ per cent; } \frac{N}{P} = \frac{1}{1.66}$$

This material was dissolved in hot ethylacetate and allowed to cool. A precipitate formed which was again dissolved in ether and precipitated by means of acetone. The substance had the following composition.

0.1008 gm. of the dry material gave 0.2170 gm. of  $\text{CO}_2$ , 0.0824 gm. of  $\text{H}_2\text{O}$ , and 0.013 gm. of ash.

0.2938 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 2.5 cc. of 0.1 N acid.

0.2888 gm. of the substance gave on fusion 0.0413 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

	C	H	N	P	$\frac{\text{N}}{\text{P}}$
Hence ash-free per cent. ....	61.33	10.08	1.22	4.15	$\frac{1}{1.54}$

The material obtained by the hydrochloric acid process gave two fractions, one formed on the precipitation of the ethereal solution by 95 per cent alcohol ( $\text{A}_1$ ) and the other by concentrating the mother liquor and precipitating it with acetone ( $\text{B}_1$ ). Fraction ( $\text{A}_1$ ) was dissolved in ether and centrifugalized to remove the very small insoluble part. The clear filtrate was concentrated and precipitated with acetone. It had the following composition.

0.2943 gm. of the substance gave on fusion 0.0424 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

0.2530 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 3.7 cc. of 0.1 N acid.

$$\text{N} = 2.04 \text{ per cent and P} = 4.07 \text{ per cent; } \frac{\text{NH}_2\text{N}}{\text{Total N}} = \frac{70}{100}$$

Fraction ( $\text{B}_1$ ) had the following composition.

0.2520 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 2.9 cc. of 0.1 N acid.

0.2503 gm. of the substance gave on fusion 0.0370 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

$$\text{N} = 1.61 \text{ per cent and P} = 4.13 \text{ per cent; } \frac{\text{NH}_2\text{N}}{\text{Total N}} = \frac{44}{100}$$

### *Reduction of Fraction A.*

It was found by experiment that the so called cuorin absorbed hydrogen with greater readiness, if prior to reduction it was purified by the hydrochloric acid process. For this reason all material used for reduction was purified in this manner. The material was dissolved in ether in 10 gm. lots, to the solution a little glacial acetic acid was added, and the catalyst was taken up in a few cc. of water and introduced into the apparatus. The reaction proceeded about 50 hours.

In the course of the reduction some solid material settled out. The reaction product was placed at 0°C. over night and filtered. The residue was dissolved in boiling 95 per cent alcohol and allowed to cool. A slightly colored precipitate is thus formed. This is dissolved in chloroform (using 1 cc. of chloroform for 0.5 gm. of the substance) and the solution poured into 5 volumes of 99.5 per cent alcohol. The precipitate thus formed is redissolved and reprecipitated twice. The final substance had the following composition.

0.1020 gm. of substance gave 0.2088 gm. of CO<sub>2</sub>, 0.0796 gm. of H<sub>2</sub>O, and 0.0152 gm. of ash.

0.1468 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 1.6 cc. of 0.1 N acid.

0.1493 gm. of the substance on fusion gave 0.0204 gm. of Mg<sub>2</sub>P<sub>2</sub>O<sub>7</sub>.

	C	H	N	P	$\frac{\text{NH}_2\text{N}}{\text{Total N}}$
Found per cent. ....	59.47	9.41	1.68	4.17	$\frac{92}{100}$

#### *Fatty Acids in This Material.*

4.8 gm. of the substance were taken up in 80.0 cc. of 3 per cent sulfuric acid and heated in a sealed tube with shaking for 24 hours at 105°C. At the end of the reaction the fatty acid was extracted with ether and the ethereal solution evaporated to dryness. The residue was extracted with acetone. The filtrate was again concentrated to a solid cake. Dried to constant weight it contained 3.08 gm. Hence the proportion of fatty acids in the material was  $\frac{3.08}{4.8} = \frac{64.16}{100}$ .

For identification of the fatty acids 15.0 gm. of the material were hydrolyzed in the same manner as above, and the fatty acids converted into the barium salts under the conditions given by Levene and Meyer.<sup>6</sup> The barium salts were extracted with ether, in which the barium salts of unsaturated fatty acids are soluble. However, the ethereal extract on evaporation left practically no residue. The barium salts were then converted into the free acids, these converted into the lead salts which were again converted into the free acids.

<sup>6</sup> Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1917, xxxi, 631.

and the free acids recrystallized out of acetone. The acid had the melting point, M. P. = 68–69° (corrected) and had the following composition.

0.0992 gm. of substance gave on combustion 0.2765 gm. of CO<sub>2</sub> and 0.1106 gm. of H<sub>2</sub>O.

	Calculated for C <sub>18</sub> H <sub>32</sub> O <sub>2</sub> . per cent	Found. per cent
C.....	76.06	76.00
H.....	12.68	12.48

The molecular weight of the substance was the following.

0.6032 gm. of substance required for neutralization 21.1 cc. of 0.1 N acid; M. P. = 285°.

#### *Glycerol Estimation.*

As in the previous work the Zeisel-Fanto<sup>7</sup> method was employed for the estimation of glycerol. The temperature of the oil bath was maintained at 125–128°C.

0.3153 gm. of the material gave 0.0940 gm. of AgT which corresponds to 11.7 per cent of glycerol. The theory for cephalin requires 12.32 per cent and for the monoacyl derivative 19.12 per cent. Levene and West found for cephalin 10 per cent, and Foster<sup>8</sup> 8.21 per cent. The method can scarcely be regarded as quantitative, but the value for glycerol obtained by us on material with a lower content of carbon is higher than the values obtained on cephalin with a higher carbon content. This result is to be expected in the light of the assumption made by us for the constitution of the so called cephalin.

#### *Fractionation of the Reduced Material.*

In a second experiment the reduced material, which had settled on cooling of a hot solution in 95 per cent alcohol, was fractionated by means of 99.5 per cent alcohol.

One part (a) remained insoluble in hot 99.5 per cent alcohol and the other (b) remained in solution in that solvent but settled out on cooling.

<sup>7</sup> Zeisel, S., and Fanto, R., *Z. anal. Chem.*, 1903, xlii, 549.

<sup>8</sup> Foster, M. L., *J. Biol. Chem.*, 1915, xx, 403.



Each one of the two fractions was then purified by dissolving in chloroform (1 gm. in 2 cc.) and pouring into three volumes of 99.5 per cent alcohol. Fraction (*a*) had the following composition.

0.1028 gm. of the substance gave 0.1962 gm. of  $\text{CO}_2$ , 0.0760 gm. of  $\text{H}_2\text{O}$ , and 0.0190 gm. of ash.

0.1445 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 1.44 cc. of 0.1 N acid.

0.1498 gm. of the substance gave on fusion 0.0329 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

	C	H	N	P
Hence ash-free per cent. ....	54.43	8.70	1.46	6.42

Fraction (*b*) had the following composition.

0.1006 gm. of the substance gave 0.2222 gm. of  $\text{CO}_2$ , 0.0816 gm. of  $\text{H}_2\text{O}$ , and 0.0124 gm. of ash.

0.1594 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 2.25 cc. of 0.1 N acid.

0.1562 gm. of the substance gave on fusion 0.025 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

	C	H	N	P	$\frac{\text{NH}_2\text{N}}{\text{Total N}}$
Hence ash-free per cent. ....	62.13	9.36	2.04	4.17	$\frac{90}{100}$

In a third experiment the fractionation was carried out by means of butyl alcohol. The material used on this occasion was the fraction soluble in hot 95 per cent alcohol and insoluble in the cold.

The substance was dissolved in boiling butyl alcohol and allowed to cool at room temperature (about 25–28°C.) when a sediment ( $a_1$ ) formed. The mother liquor on standing at about 10°C. gave rise to a second precipitate ( $b_1$ ). ( $a_1$ ) had the following composition.

0.1028 gm. of the substance gave 0.2278 gm. of  $\text{CO}_2$ , 0.0910 gm. of  $\text{H}_2\text{O}$ , and 0.0130 gm. of ash.

0.1506 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 2.1 cc. of 0.1 N acid.

0.1504 gm. of the substance gave on fusion 0.0274 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

	C	H	N	P	$\frac{\text{NH}_2\text{N}}{\text{Total N}}$
Hence ash-free per cent. ....	61.03	10.11	2.00	5.15	$\frac{60}{100}$

( $b_1$ ) contained the elements of nitrogen and phosphorus in the following proportions.

0.1690 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 2.55 cc. of 0.1 N acid.

0.1647 gm. of the substance gave on fusion 0.0196 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

	N	P	$\frac{\text{NH}_2\text{N}}{\text{Total N}}$
Hence found per cent.....	2.11	3.33	$\frac{30}{100}$

( $a_1$ ) on renewed purification through butyl alcohol again gave two fractions, the less soluble ( $a_2$ ) and the more soluble ( $b_2$ ). ( $a_2$ ) had the following composition.

0.1022 gm. of the substance gave 0.206 gm. of  $\text{CO}_2$ , 0.0838 gm. of  $\text{H}_2\text{O}$ , and 0.016° gm. of ash.

0.1430 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 2.25 cc. of 0.1 N acid.

0.1367 gm. of the substance gave on fusion 0.0266 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

	C	H	N	P	$\frac{\text{NH}_2\text{N}}{\text{Total N}}$
Hence ash-free per cent.....	61.94	9.47	2.43	5.81	$\frac{85}{100}$

( $b_2$ ) gave the following analytical values.

0.1008 gm. of the substance gave 0.2172 gm. of  $\text{CO}_2$ , 0.1172 gm. of  $\text{H}_2\text{O}$ , and 0.0126 gm. of ash.

0.1474 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 2.2 cc. of 0.1 N acid.

0.1512 gm. of the substance gave on fusion 0.0290 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

	C	H	N	P	$\frac{\text{NH}_2\text{N}}{\text{Total N}}$
Hence ash-free per cent.....	59.19	12.92	2.09	5.41	$\frac{78}{100}$

All the crude reduced material when it is taken up in hot 95 per cent alcohol leaves a small insoluble residue which adheres to the palladium tenaciously. It was found possible to separate it from the palladium by means of a hot solution of the following solvent: 1 part by volume of chloroform, 1 part of ether, and 1 part of 95 per cent alcohol. On standing at room temperature it formed a precipitate ( $a_3$ ) and filtrate ( $b_3$ ). ( $a_3$ ) was dissolved in chloroform and precipitated by means of three volumes of 99.5 per cent alcohol. The substance obtained in this manner had the following composition.

0.0998 gm. of the substance gave 0.2274 gm. of  $\text{CO}_2$ , 0.0810 gm. of  $\text{H}_2\text{O}$ , and 0.0134 gm. of ash.

0.1350 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 1.6 cc. of 0.1 N acid.

0.1540 gm. of the substance gave on fusion 0.0211 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

	C	H	N	P	$\frac{\text{NH}_3\text{N}}{\text{Total N}}$
Hence ash-free per cent. ....	62.51	9.68	1.58	4.01	$\frac{95}{100}$

## d-CHONDROSAMINO- AND d-CHITOSAMINOHEPTONIC ACIDS.

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The theoretical interest attached to the preparation of these substances was discussed in previous communications.<sup>1</sup> The behavior of them to nitrous acid, it is hoped, may furnish valuable information in connection with the problem of the allocation of the amino group in the  $\alpha$ -aminohexoses and  $\alpha$ -aminohexosaminic acids.

As regards the preparation of the heptonic acids, the present communication contains specific directions leading to satisfactory yields of chondrosaminoheptonic acid, and simplified directions, as compared with the original, for the preparation of chitosaminoheptonic acids. Since the product obtained on the action of prussic acid on hexosamines is a mixture of two epimers, it became necessary to separate the mixture into its components. This part of the problem was solved satisfactorily.

The *d*-chondrosaminoheptonic acids with the original specific rotation of  $[\alpha]_D^{25} = -3.5$  were fractioned into a levo-*d*-chondrosaminoheptonic acid, with the initial rotation  $[\alpha]_D^{19} = -8.25$  and with the equilibrium value of  $[\alpha]_D^{25} = -13.00$  and a dextro-*d*-chondrosaminoheptonic acid with a rotation of  $[\alpha]_D^{25} = +42.5$  and equilibrium rotation of  $[\alpha]_D^{25} = +65.0$ . In the original product the levo form predominates.

The *d*-chitosaminoheptonic acids with the rotation  $[\alpha]_D^{30} = +4.0$  and equilibrium value of  $[\alpha]_D^{30} = -1.0$  were separated into a dextro-*d*-chitosaminic acid with initial rotation of  $[\alpha]_D^{25} = +6.5$  and equilibrium rotation of  $[\alpha]_D^{25} = +2.75$  and a levo-*d*-chitosaminic acid with initial rotation of  $[\alpha]_D^{25} = -7.5$  and equilibrium rotation of  $[\alpha]_D^{25} = -12.0$ . The dextro form predominates in the original product.

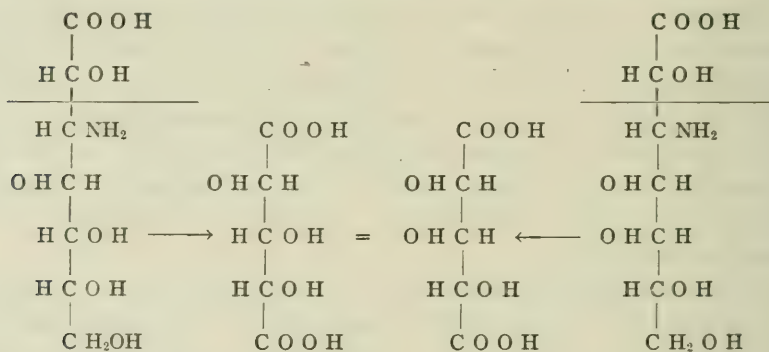
<sup>1</sup> Levene, P. A., *J. Biol. Chem.*, 1916, xxiv, 55; xxvi, 152.



The problem of the action of nitrous acid is as yet not brought to a conclusion and is reported here only because one of the authors is compelled to discontinue his cooperation.

It was attempted first to isolate the diaminoheptonic acids, but thus far they were not obtained in a satisfactory physical condition.

On action with nitric acid subsequent to deamination insoluble calcium salts were obtained which gave analytical data satisfactory for the calcium salts of trioxylglutaric acids. Thus: Repeated recrystallization, conversion into lead salt with reversion into calcium salt, fractionation with lead acetate, and subsequent conversion into calcium salt all led to calcium salts with the values of the elements as required for trioxylglutaric acids. Yet such a conclusion is not acceptable for the reason that both heptonic acids should yield one and the same trioxylglutaric acid, as seen from the following.



Whereas the calcium salts obtained from the two acids possessed a different optical rotation, the rotation of the acid obtained from chondrosaminoheptonic was  $[\alpha]_D^{25} = +5.0$  initial and  $[\alpha]_D^{25} = +1.5$  equilibrium. That of the chitosaminoheptonic  $[\alpha]_D^{25} = +10.0$  initial and  $[\alpha]_D^{25} = +17.0$  equilibrium. Hence, conditions will have to be discovered for the purification of the dicarboxyl acids arising from diaminoheptonic acids.

## EXPERIMENTAL.

*Preparation of Chondrosaminoheptonic Acid.*

35 gm. of chondrosamine hydrochloride were taken up in 70 cc. of water; 35 cc. of 80 per cent aqueous solution of hydrocyanic acid and 22 cc. of strong ammonia water were added. This mixture was warmed between 42–45° for about 15 minutes. The solution turned dark in color and became viscous. It was cooled in an ice-alcohol bath until the temperature of solution came down to 0°C., when it was transformed into an aqueous solution of 200 gm. of barium hydroxide. The solution was allowed to stand about 1 hour, then boiled on free flame about 3 hours, and distilled under diminished pressure to dryness. The residue was dissolved in 100 cc. of water and the solution was again distilled to dryness; this operation was repeated ten times. By this procedure all ammonia was driven off.

Barium was removed by means of a small excess of sulfuric acid, and the excess of this was removed by means of lead carbonate. The hydrochloric acid was removed by means of  $\text{AgCO}_3$ . The excess of lead and silver was removed by means of hydrogen sulfide gas. The filtrate from silver sulfide was concentrated under diminished pressure to a syrup, and seeded with chondrosaminoheptonic acid. Hot methyl alcohol was added very cautiously until slight opalescence and the solution was allowed to stand about 72 hours at room temperature. (Sometimes the substance crystallized out of aqueous solution.)

The crystalline deposit was filtered, washed with 50 per cent alcohol, 95 per cent alcohol, and absolute alcohol successively, and finally with ether. The best yield was 11 gm. In this manner 443 gm. of the substance were prepared.

*Levo-d-Chondrosaminoheptonic Acid.*

440 gm. of chondrosaminoheptonic acid, which had the following rotation in 2.5 per cent HCl solution

$$[\alpha]_D^{25} = \frac{\text{Initial, } -0.14 \times 100}{1 \times 4} = -3.5$$

were dissolved in 800 cc. of hot water. On standing over night crystals separated out, which had the following rotation.

Initial.	Equilibrium (24 hours).
$[\alpha]_D^{25} = \frac{-0.30 \times 100}{1 \times 4} = -7.5$	$[\alpha]_D^{25} = \frac{-0.48 \times 100}{1 \times 4} = -12.00$

Yield was 344 gm.

The 344 gm. of heptonic acid were dissolved in 800 cc. of hot water and 391 gm. of crystals were obtained. The rotation in 2.5 per cent HCl solution was the following.

Initial.	Final (24 hours).
$[\alpha]_D^{25} = \frac{-0.33 \times 100}{1 \times 4} = -8.25$	$[\alpha]_D^{25} = \frac{-0.49 \times 100}{1 \times 4} = -12.25$

These crystals were dissolved again in 800 cc. of hot water and 265 gm. of crystals were obtained which had the same rotation in 2.5 per cent HCl solution.

Initial.	Final (24 hours).
$[\alpha]_D^{25} = \frac{-0.33 \times 100}{1 \times 4} = -8.25$	$[\alpha]_D^{25} = \frac{-0.52 \times 100}{1 \times 4} = -13.00$

In the mother liquor a further deposit of the same substance settled out. The total yield of levo-chondrosaminoheptonic acid was 313 gm. It crystallized out of water in elongated prisms. Melting point was M. P. = 139°C. (corrected) with decomposition.

#### *Dextro-d-Chondrosaminoheptonic Acid.*

The mother liquor of the first separation was concentrated under diminished pressure and a precipitate formed and was filtered off. To the filtrate an equal volume of 95 per cent alcohol was added and the solution was allowed to stand 24 hours. A second precipitate formed and was filtered off again and the solution was poured into absolute alcohol under constant stirring. A gummy precipitate formed which was removed and the solution was concentrated under diminished pressure at room temperature to a syrup. Alcohol was then added and the solution allowed to stand for several days.

Crystals separated which had the following rotation in 2.5 per cent HCl solution

Initial.	Final (24 hours).
No. 351 $[\alpha]_D^{25} = \frac{+0.64 \times 100}{1 \times 4} = +16.0$	$[\alpha]_D^{25} = \frac{+1.02 \times 100}{1 \times 4} = +25.50$

Yield was 1.9 gm.

In the mother liquor a second deposit of 2.3 gm. of crystals formed which had the following rotation in 2.5 per cent HCl solution.

Initial.	Final (24 hours).
No. 311	
$[\alpha]_D^{25} = \frac{+0.50 \times 100}{1 \times 2} = +25.0$	$[\alpha]_D^{25} = \frac{+0.85 \times 100}{1 \times 2} = +42.5$

In the mother liquor a further deposit of crystals formed which had the following rotation in 2.5 per cent HCl solution.

Initial.	Final (24 hours).
No. 352	
$[\alpha]_D^{25} = \frac{+0.85 \times 100}{1 \times 2} = +42.5$	$[\alpha]_D^{25} = \frac{+1.43 \times 100}{1 \times 2} = +71.50$

From the mother liquor of this substance there was obtained on further crystallization a substance with the following specific rotation in 2.5 per cent HCl solution.

Initial.	Final (24 hours).
No. 407	
$[\alpha]_D^{25} = \frac{+0.85 \times 100}{1 \times 2} = +42.5$	$[\alpha]_D^{25} = \frac{+1.30 \times 100}{1 \times 2} = +65.0$

### *Preparation of Chitosaminoheptonic Acids.*

The conditions for the preparation of these acids are more uncertain than of any other substance of this group. At one time, namely in 1915, good yields of the crystalline substance were obtained by one of us in the following way. 50.0 gm. of chitosamine hydrochloride were taken up in 100 cc. of water, 18 cc. of an 80 per cent aqueous prussic acid and 25 cc. of ammonium hydroxide were added, and the solution allowed to stand from 24 to 48 hours. The product was then transferred into water containing about 150.0 gm. of barium hydroxide and the solution was boiled over free flame as long as ammonia was still evolved (about 48 hours). The barium and hydrochloric acids were removed in the usual way and the final solution was concentrated under diminished pressure to a thick syrup. This was taken up in a little water, hot 95 per cent alcohol was added as long as a gummy precipitate formed, and the supernatant liquid was decanted and allowed to stand over night at 0°C. A crystalline deposit formed and from the mother liquor on concentration and repeated treatment



with alcohol, etc., a second crop of crystals was generally obtained. The total yield was about 30 per cent of the employed chitosamine. This material was slightly levorotary.

In 1916 the laboratories were transferred into a new building and all attempts to prepare the material by apparently the same process failed. After many experiments it was finally possible again to obtain the substance under the following conditions. 35 gm. of chitosamine hydrochloride were dissolved in 100 cc. of water; 35 gm. of aqueous 80 per cent prussic acid and 20 cc. of ammonia were added. The mixture was warmed to 30°C. and then allowed to stand at room temperature for 1 hour. The temperature generally remained at that temperature and all the sugar was dissolved in the course of that time. The solution was then transferred into an aqueous solution of barium hydroxide and the further treatment proceeded as above. The final solution was concentrated to a small volume and hot methyl alcohol was added to slight opalescence. The solution was allowed to stand at room temperature over night. A crystalline deposit formed, the yield being about 15 per cent of the employed chitosamine hydrochloride. The specific rotation of this substance was  $[\alpha]_D^{20} = +4.10$ .

*Dextro-d-Chitosaminoheptonic Acid.*

130 gm. of chitosaminoheptonic acid were dissolved in four parts of hot water. The original material had the following rotation in 2.5 per cent HCl solution.

	Initial.	Final.
No. 225		
	$[\alpha]_D^{20} = \frac{+0.16 \times 100}{1 \times 4} = +4.0$	$[\alpha]_D^{20} = \frac{-0.04 \times 100}{1 \times 4} = -1.0$

After standing over night no crystals separated, hence the solution was concentrated under diminished pressure until a crystalline deposit began to form and then allowed to stand over night. 7½ gm. of crystals were obtained which had the following rotation in 2.5 per cent HCl solution.

	Initial.
No. 236	
	$[\alpha]_D^{25} = \frac{+0.23 \times 100}{1 \times 4} = +5.75$

These 74 gm. of heptonic acid were dissolved in 150 cc. of hot water; 43 gm. of crystals settled out which had the following rotation in 2.5 per cent HCl solution.

Initial.	Final (24 hours).
No. 246	
$[\alpha]_D^{25} = \frac{+0.26 \times 100}{1 \times 4} = +6.5$	$[\alpha]_D^{25} = \frac{+0.11 \times 100}{1 \times 4} = +2.75$

These crystals were dissolved again in 86 cc. of hot water and there were obtained 35 gm. of crystals which had the following rotation in 2.5 per cent HCl solution.

Initial.
No. 247
$[\alpha]_D^{25} = \frac{+0.26 \times 100}{1 \times 4} = +6.5$

These 35 gm. were again dissolved in 70 cc. of hot water, and there were obtained 28 gm. of crystals which had the following rotation in 2.5 per cent HCl solution.

Initial.
$[\alpha]_D^{25} = \frac{+0.26 \times 100}{1 \times 4} = +6.5$

From the mother liquor 37 gm. of the same substance were obtained; the total yield was 65 gm. The substance crystallized out of water in heavy prisms. Melting point was M. P. = 192°C. with decomposition.

### *Levo-d-Chitosaminoheptonic Acid.*

To the mother liquor of the first precipitate hot methyl alcohol was added to slight opalescence and the solution was allowed to stand 4 hours. A precipitate then separated and was filtered off; the mother liquor was concentrated under diminished pressure and to it hot alcohol was added. A precipitate was again formed and filtered off and to the mother liquor more alcohol was added. 5 gm. of crystals separated which had the following rotation in 2.5 per cent HCl solution.

Initial.
No. 307
$[\alpha]_D^{25} = \frac{-0.16 \times 100}{1 \times 4} = -4.0$

These 5 gm. were dissolved in 5 cc. of hot water. After long standing there were obtained 1.85 gm. of crystals which had the following rotation in 2.5 per cent HCl solution.

Initial.

No. 309

$$[\alpha]_D^{25} = \frac{-0.13 \times 100}{1 \times 2} = -6.5$$

The mother liquor of No. 307 was allowed to stand. On long standing there crystallized 4.75 gm. of a substance with the following rotation in 2.5 per cent HCl solution.

Initial.

No. 261

$$[\alpha]_D^{25} = \frac{-0.11 \times 100}{1 \times 2} = -5.5$$

These crystals were dissolved in 5 cc. of hot water and gave 1.6 gm. of crystals which had the following rotation.

Initial.

No. 358

$$[\alpha]_D^{25} = \frac{-0.15 \times 100}{1 \times 2} = -7.5$$

Nos. 309 and 358 were combined and dissolved in 4 cc. of hot water. There were obtained 1.5 gm. of crystals which had the following rotation in 2.5 per cent HCl solution.

Initial.

Final (24 hours).

No. 380

$$[\alpha]_D^{25} = \frac{-0.15 \times 100}{1 \times 2} = -7.5 \quad [\alpha]_D^{25} = \frac{-0.24 \times 100}{1 \times 2} = -12.0$$

The 1.5 gm. were dissolved again in 1.5 cc. of hot water and gave 1.2 gm. of crystals which had the following rotation in 2.5 per cent HCl solution.

Initial.

No. 381

$$[\alpha]_D^{25} = \frac{-0.15 \times 100}{1 \times 2} = -7.5$$

It crystallized out of 25 per cent alcohol solution in long prismatic needles. Melting point was M. P. = 139°C. (corrected) with decomposition.

*Oxidation of Levo-Chondroheptonic Acid with Nitric Acid.*

10.0 gm. of substance were dissolved in 70 cc. of water and 30 cc. of 10 per cent hydrochloric acid. 10 gm. of AgNO<sub>2</sub> were added and the mixture was allowed to stand over night. 4.0 gm. of AgNO<sub>2</sub>

and 4 cc. of 10 per cent HCl were then added. An excess of HCl was found, hence  $\text{AgNO}_3$  was added, filtered, then excess of silver was removed by  $\text{H}_2\text{S}$ . The filtrate was concentrated to 40 cc. under diminished pressure. An equal volume of concentrated  $\text{HNO}_3$  was added and heated over flame. It began to react after 2 minutes. The reaction was allowed to proceed 9 minutes and the product was concentrated immediately on a clock glass.

Some crystals, which proved to be oxalic acid, were filtered off and the residue was converted into a calcium salt. The yield was 0.200 gm. The mother liquor was concentrated to very small volume and allowed to crystallize. The yield was 0.800 gm. (No. 191). In 2.5 per cent HCl No. 191 had the following rotation

$$[\alpha]_D^{25} = \frac{+0.10 \times 100}{1 \times 2} = +5.0$$

#### *Purification of Calcium Salts.*

The calcium salts obtained from several experiments were suspended in boiling water and the free acid was liberated by the addition of oxalic acid. The substance was reconverted into calcium salts. The salt obtained in this manner had the following rotation

$$[\alpha]_D^{25} = \frac{\text{Initial.} \quad +0.10 \times 100}{1 \times 2} = +5.0 \quad [\alpha]_D^{25} = \frac{\text{Final.} \quad +0.07 \times 100}{1 \times 2} = +3.5$$

and gave the following analytical data.

0.0918 gm. of substance gave 0.0240 gm. of  $\text{H}_2\text{O}$ , 0.0934 gm. of  $\text{CO}_2$ , and 0.0238 gm. of ash.

	Calculated for $\text{C}_8\text{H}_{10}\text{O}_7\text{Ca}$ , per cent	Found. per cent
C.....	27.52	27.74
H.....	2.75	2.92
CaO.....	25.69	25.92

#### *Milder Oxidation of Levo-Chondrosaminoheptonic Acid with Nitric Acid.*

10 gm. of heptonic acid, 70 cc. of water, 30 cc. of hydrochloric acid, and 12 gm. of  $\text{AgNO}_2$  were allowed to stand over night at  $10^\circ\text{C}$ . 2 gm. of  $\text{AgNO}_2$  and 2 cc. of HCl were added in the evening and the



following morning the filtrate from  $\text{Ag}_2\text{S}$  was concentrated under diminished pressure without heating to about 40 cc. 10 cc. of this solution were mixed with 10 cc. of concentrated nitric acid and evaporated on a clock glass on a water bath. The residue was dissolved in a little water and evaporated again. The white residue was taken up in hot water and converted into a calcium salt in the usual way and concentrated under diminished pressure without heating until a precipitate formed (No. 299).

No. 299 had the following rotation in 2.5 per cent HCl solution.

$$\begin{array}{c} \text{Initial.} \\ [\alpha]_D^{25} = \frac{+0.10 \times 100}{1 \times 2} = +5.0 \end{array}$$

On further concentration a further precipitate, No. 300, was obtained.

10 gm. of heptonic acid were deaminized, oxidized, and converted into Ca salt just the same as in the previous experiment.

First fraction = 1.7 gm.

Second fraction = 0.5 gm.

The calcium salts obtained in this manner were combined and boiled with 500 cc. of water, the insoluble precipitate was filtered off, which had the following rotation in 2.5 per cent HCl solution.

$$\begin{array}{cc} \begin{array}{c} \text{Initial.} \\ [\alpha]_D^{25} = \frac{+0.09 \times 100}{1 \times 2} = +4.5 \end{array} & \begin{array}{c} \text{Final (24 hours).} \\ [\alpha]_D^{25} = \frac{+0.02 \times 100}{1 \times 2} = +1.0 \end{array} \end{array}$$

It analyzed as follows:

0.1036 gm. of substance gave 0.0288 gm. of  $\text{H}_2\text{O}$ , 0.1032 gm. of  $\text{CO}_2$ , and 0.0260 gm. of ash.

	Calculated for $\text{C}_8\text{H}_{15}\text{O}_7\text{Ca}$ , per cent	Found, per cent
C.....	27.52	27.16
H.....	2.75	3.11
CaO.....	25.69	25.09

The filtrate on standing gave a second precipitate (No. 325).

No. 325 had the following rotation in 2.5 per cent HCl solution.

$$\begin{array}{cc} \begin{array}{c} \text{Initial.} \\ [\alpha]_D^{25} = \frac{+0.10 \times 100}{1 \times 2} = +5.0 \end{array} & \begin{array}{c} \text{Final (24 hours).} \\ [\alpha]_D^{25} = \frac{+0.03 \times 100}{1 \times 2} = +1.5 \end{array} \end{array}$$

It analyzed as follows:

0.0984 gm. of substance gave 0.0258 gm. of H<sub>2</sub>O, 0.0998 gm. of CO<sub>2</sub>, and 0.0246 gm. of ash.

	Calculated for C <sub>3</sub> H <sub>5</sub> O <sub>7</sub> Ca. <i>per cent</i>	Found. <i>per cent</i>
C.....	27.52	27.65
H.....	2.75	2.93
CaO.....	25.69	25.00

The mother liquor of No. 325 was concentrated under diminished pressure until crystallization set in and was then allowed to stand in the cold. A further precipitation was obtained, which had the following rotation in 2.5 per cent HCl solution.

$$[\alpha]_D^{25} = \frac{+0.10 \times 100}{1 \times 2} = +5.0$$

It analyzed as follows:

0.1034 gm. of substance gave 0.0308 gm. of H<sub>2</sub>O, 0.1058 gm. of CO<sub>2</sub>, and 0.0250 gm. of ash.

	Calculated for C <sub>3</sub> H <sub>5</sub> O <sub>7</sub> Ca. <i>per cent</i>	Found. <i>per cent</i>
C.....	27.52	27.90
H.....	2.75	3.33
CaO.....	25.69	24.28

Several samples were combined. The total weight was 8.0 gm. These were suspended in 200 cc. of water and boiled. The first precipitate was removed by filtration.

To the mother liquor neutral lead acetate was added and a precipitate was obtained which was decomposed by H<sub>2</sub>S and concentrated under diminished pressure to nearly dryness. The residue was extracted with acetone. The acetone was evaporated to small volume and allowed to stand at room temperature. As it remained syrup it was placed in a vacuum desiccator over H<sub>2</sub>SO<sub>4</sub>. On long standing it showed a tendency to crystallize. However, crystallization proceeded very slowly, hence it was transformed into the calcium salt (No. 403). No. 403 was analyzed as follows:

0.1016 gm. of substance gave 0.0278 gm. of H<sub>2</sub>O, 0.1050 gm. of CO<sub>2</sub>, and 0.0261 gm. of ash.

	Calculated for C <sub>8</sub> H <sub>16</sub> O <sub>7</sub> Ca. per cent	Found. per cent
C.....	27.52	28.18
H.....	2.75	3.05
CaO.....	25.69	25.68

From the mother liquor a colorless precipitate was obtained (No. 438). No. 438 had the following rotation in 2.5 per cent HCl solution.

$$[\alpha]_D^{25} = \frac{\text{Initial.} \quad +0.10 \times 100}{1 \times 2} = +5.0 \quad [\alpha]_D^{25} = \frac{\text{Final (24 hours).} \quad +0.10 \times 100}{1 \times 2} = +5.0$$

It analyzed as follows:

0.0979 gm. of substance gave 0.0244 gm. of H<sub>2</sub>O, 0.0992 gm. of CO<sub>2</sub>, and 0.0252 gm. of ash.

	Calculated for C <sub>8</sub> H <sub>16</sub> O <sub>7</sub> Ca. per cent	Found. per cent
C.....	27.52	27.63
H.....	2.75	2.78
CaO.....	25.69	25.73

#### *Oxidation of Dextro-Chitosaminoheptonic Acid with Nitric Acid.*

10 gm. of heptonic acid, 70 cc. of water, 30 cc. of 10 per cent hydrochloric acid, and 12 gm. of AgNO<sub>2</sub> were allowed to stand over night in the cold room. Two portions of 2 gm. of AgNO<sub>2</sub> and 2 cc. of 10 per cent HCl were added. Further treatment was the same as in the previous experiments.

Three preparations, No. 314, first fraction = 1.25 gm., No. 315, second fraction = 1.05 gm., No. 316, third fraction = 10 gm. of heptonic acid, were treated just the same as previous experiments and 1.75 gm. of calcium salt were obtained.

#### *Purification of the Ca Salt.*

All the Ca salts of chitosaminoheptonic acid were combined and boiled with 300 cc. of water. A small part remained insoluble (No. 341). This analyzed as follows:

0.1043 gm. of substance gave 0.0264 gm. of H<sub>2</sub>O, 0.1032 gm. of CO<sub>2</sub>, and 0.0285 gm. of ash.

	Calculated for $C_8H_8O_7Ca$ , per cent	Found. per cent
C.....	27.52	26.98
H.....	2.75	2.83
CaO.....	25.69	27.32

The mother liquor on standing formed a precipitate which had the following rotation in 2.5 per cent HCl solution.

$$[\alpha]_D^{25} = \frac{\text{Initial.}}{1 \times 2} = +10.0 \quad [\alpha]_D^{25} = \frac{\text{Final.}}{1 \times 2} = +17.0$$

It analyzed as follows:

0.0996 gm. of substance gave 0.0290 gm. of  $H_2O$ , 0.1062 gm. of  $CO_2$ , and 0.0230 gm. of ash.

	Calculated for $C_8H_8O_7Ca$ , per cent	Found. per cent
C.....	27.52	29.07
H.....	2.75	3.25
CaO.....	25.69	23.29

From the mother liquor on further standing a further precipitate was obtained which had the following rotation in 2.5 per cent HCl solution.

$$[\alpha]_D^{25} = \frac{\text{Initial.}}{1 \times 2} = +8.0 \quad [\alpha]_D^{25} = \frac{\text{Final.}}{1 \times 2} = +15.0$$

It analyzed as follows:

0.1004 gm. of substance gave 0.0274 gm. of  $H_2O$ , 0.1034 gm. of  $CO_2$ , and 0.0238 gm. of ash.

	Calculated for $C_8H_8O_7Ca$ , per cent	Found. per cent
C.....	27.52	28.47
H.....	2.75	3.09
CaO.....	25.69	23.09

All calcium salts were combined; the total weight was 2.9 gm. They were dissolved in 500 cc. of hot water, 1.0 gm. of oxalic acid was added, and the solution boiled until all the calcium was removed. To the filtrate neutral lead acetate was added and the precipitate thus formed was decomposed by  $H_2S$  and reconverted into the calcium salt (No. 421). It analyzed as follows:



0.1047 gm. of substance gave 0.0276 gm. of H<sub>2</sub>O, 0.1060 gm. of CO<sub>2</sub>, and 0.0264 gm. of ash.

	Calculated for C <sub>8</sub> H <sub>6</sub> O <sub>7</sub> Ca. <i>per cent</i>	Found. <i>per cent</i>
C.....	27.52	27.60
H.....	2.75	2.94
CaO.....	25.69	25.21

From the mother liquor of No. 421 on concentration a further precipitate was formed. It was extracted with boiling glacial acetic acid twice, washed with alcohol and ether, and dried. It had the following rotation in 2.5 per cent HCl solution.

$$\begin{array}{cc}
 \text{Initial.} & \text{Final (24 hours).} \\
 [\alpha]_D^{25} = \frac{+0.17 \times 100}{1 \times 2} = +8.5 & [\alpha]_D^{25} = \frac{+0.29 \times 100}{1 \times 2} = +14.5
 \end{array}$$

It analyzed as follows:

0.1104 gm. of substance gave 0.0288 gm. of H<sub>2</sub>O, 0.1136 gm. of CO<sub>2</sub>, and 0.0280 gm. of ash.

	Calculated for C <sub>8</sub> H <sub>6</sub> O <sub>7</sub> Ca. <i>per cent</i>	Found. <i>per cent</i>
C.....	27.52	28.06
H.....	2.75	2.91
CaO.....	25.69	25.36

## A FERMENTATION PROCESS FOR THE PRODUCTION OF ACETONE AND ETHYL ALCOHOL.<sup>1,2,3</sup>

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Acetone was one of the substances for which the war created a greatly increased demand. It was needed by all the Allies for the dope used on airplane wings and by the English in addition for the manufacture of cordite. The ordinary source of acetone, the dry distillation of wood, proved quite inadequate to supply the quantities needed. It became necessary, therefore, to develop some new method for the production of acetone. Large quantities were made from calcium acetate which was in turn produced from acetic acid obtained by the oxidation of alcohol. The expense of this process, however, rendered it impracticable. It seemed important, therefore, under these conditions, to attempt the development of a direct fermentation process for the production of acetone, inasmuch as such a method,

<sup>1</sup> The work described in this paper is the outcome of a suggestion of the Council for National Defense that a fermentation process for the production of acetone be worked out. The laboratory work and the preliminary large scale experiments were conducted at the chemical laboratories of The Rockefeller Institute. A second series of experiments was done at the laboratory of Arthur D. Little, Inc., Cambridge, Mass., under a grant from the Bureau of Aircraft Production, and the final work was carried out at Terre Haute, Ind., at the plant of the Commercial Solvents Corporation. The authors wish to express their indebtedness to the Commercial Solvents Corporation, and particularly to Dr. Nelson B. Mayer and Mr. Robert D. Clark, for placing every facility of the plant at Terre Haute at their disposal.

<sup>2</sup> Published by permission of the Director, Chemical Warfare Service.

<sup>3</sup> The process described in this paper is protected by U. S. Pat. 1,293,172, assigned to The Rockefeller Institute for Medical Research. This patent has been dedicated to public use and is held, under these conditions, by the U. S. Patent Office.

if successfully developed, would furnish acetone in practically unlimited quantity and at a low cost.

Several fermentation processes for the production of acetone have been described and patented,<sup>1</sup> and of these, the Fernbach process,<sup>2</sup> has proved to be a commercial success. It has the disadvantage however, of producing twice as much butyl alcohol as acetone. This alcohol, though valuable, is one for which there is a limited demand and is therefore difficult to dispose of when produced in large quantity. The present work is an attempt to develop a process which yielded largely ethyl alcohol as a by-product, with smaller amounts of propyl and butyl alcohols.

The organism used is described under the name of *B. acetoethylicum* in a paper which is to appear shortly in the *Journal of Biological Chemistry*. The general characteristics of the organism together with the optimum conditions for its growth may be summarized as follows:

TABLE I.

DESCRIPTION OF THE ORGANISM—Described according to descriptive chart of the Society of American Bacteriologists.

SOURCE—From old potatoes obtained from Berkshire Co., Mass., July 1, 1917.

PROPOSED NAME—*Bacillus acetoethylicum*.

#### I—MORPHOLOGY

(1) Vegetative cells, motile. From 24 hrs. agar slant, 40° C.—Short rods 4–6 $\mu$ $\times$ 0.2–0.3 $\mu$ . No chains. Ends rounded. Stain evenly with Loeffler's methylene blue or gentian violet. Gram negative.	From 24 hrs. 10 per cent corn media—Same as above but occasional short chains. From old (6–10 days) 10 per cent corn media—Stain unevenly with deeply staining spot at end or in center.	(2) Spores — Elliptical, form at end of rods. Stain easily with methylene blue or gentian violet. 0.5–1.0 $\mu$ in diameter.
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<sup>1</sup> Bayer and Company, D. R. P. 283,107, July 1913; D. R. P. 291,162, Jan. 1914; Brit. Pat. 14,371, June 15, 1914; Delbrück, U. S. Pat. 1,169,321; Fernbach and Strange, U. S. Pat. 1,044,368, Nov. 12, 1912.

<sup>2</sup> A process similar at least to the original Fernbach process has been developed in England by Dr. Weissman. It has been used successfully in this country and in Canada.

## II—CULTURAL FEATURES

- (1) *2 per cent glucose agar slant, 24 hrs., 40° C.*—Moderate, spreading, effuse, dull, translucent, no odor. Condensation water opaque.
- (2) *Potato, 24 hrs., 40° C.*—Gas bubbles all over media. Crumbles easily. No odor. 2-3 days, 40° C.—Media sinks to grayish white paste.
- (3) *Glucose broth, 24 hrs., 40° C.*—Cloudy. No odor. 2-3 days—Shiny mass in bottom.
- (4) *Lilmus milk, 24 hrs., 40° C.*—Bottom of tube white, no gas, odor, acid, or clot. 36 hrs., 40° C.—Milk red on top, rest white. 72 hrs., 40° C.—Same but coagulated; clot does not digest subsequently.
- (5) *Agar plate colonies, 2 per cent glucose agar, 24 hrs., 40° C.*—Growth slowly spreading. Round outline irregular. Surface smooth. Elevation effuse.
- Edge entire or undulate. Internal structure, coarsely granular.
- (6) *Sodium chloride in bouillon*—Inhibiting concentration 4-5 per cent.
- (7) *Nitrogen—With sugar as carbohydrate* obtained from peptone, proteins, or ammonium salts.
- With starch*—Same but cannot use ammonium salts.
- (8) *Best media for long-continued growth*—2 per cent corn in media with  $\text{CaCO}_3$ .

## III—OPTIMUM REACTION OF MEDIA

For growth,  $\text{pH} = 8.0$   
to 9.0.

For fermentation,  
 $\text{pH} = 6.0$  to 8.0.

## IV—VITALITY OF CULTURE MEDIA

At least 6 months at  
room temperature.

At least 1 month at  
40° C.

## V—TEMPERATURE RELATION

Optimum temperature  
40° to 43° C.

Spores may be boiled  
at least 20 min.

## VI—RESISTANT TO DRYING

## VII—PRODUCTS OF REACTION

Formic acid

Ethyl, propyl, butyl  
alcohol

Acetone

## VIII—PATHOGENICITY

Non-pathogenic to mice



## IX—FERMENTATION OF SUGARS, ETC.

Ferments the following sugars in 1 per cent solution with addition of  $\text{CaCO}_3$  and peptone or nitrogen source, 15 cc. in test tubes.

Temperature =  $37^\circ \text{C}$ ., Substrate = 2 per cent Sugar, 1 per cent Peptone, 2 per cent  $\text{CaCO}_3$ . Time of Fermentation = 10 Days

SUBSTANCE	Acetone Per cent	Alcohol Per cent	SUBSTANCE	Acetone Per cent	Alcohol Per cent
Galactose.....	4- 5	22-24	Dextrin.....	6- 7	14-16
Maltose.....	6- 7	23-24	Dextrose.....	9-10	22-23
Mannose.....	6- 7	22-23	Levulose.....	8-10	24-25
Raffinose.....	8-10	22-23	Xylose.....	4- 5	18-20
<i>d</i> -Arabinose.....	6- 7	12-16	Glycerin.....	..	40-43
Calcium lactate.....	..	..	Sucrose.....	8- 9	24-26
Starch.....	8-10	20-24			

Ferments levulose and galactose under following conditions:

Medium: 1 g.  $\text{KH}_2\text{PO}_4$ ,  
1 g.  $(\text{NH}_4)_2\text{HPO}_4$ ,  
0.01 g.  $\text{NaCl}$ , 1.0 g.  
 $\text{CaCO}_3$ , 10.0 g. levu-

lose, per liter. Put in tubes and sterilized as described by Schar-dinger; inoculated with pure culture of the bacteria and incubated 12 days at  $40^\circ\text{C}$ .

Acetone = 8-9 per cent.  
Alcohol = 14-20 per cent.

Starch does not ferment under these conditions.

## X—AIR RELATION

Facultative anaerobe.

## XI—SLIME FORMATION

In 10 per cent sugar solutions, having a reaction of  $\text{pH}$  8.0-9.0, large quantities of

slime are formed so that the whole media becomes very viscous. Under conditions of

fermentation small deposit of slime settles to the bottom.

It was found in the preliminary laboratory experiments already described that the fermentation, if carried out in the usual way by inoculating a sterile mash with a relatively small inoculating culture, required 5 to 6 days for completion. It was also found that the organisms collected in a slimy mass at the bottom of the fermenting liquid, a fact which becomes more disadvantageous with the increase in the size of the fermenting vessel. Both these difficulties were overcome by using a fermenter filled with pieces of broken marble.<sup>1</sup>

<sup>1</sup> It was found later that any inert material could be used, provided the fresh mash was made slightly alkaline ( $\text{pH}$  8.0 to 9.0), so as to maintain the reaction at a  $\text{pH}$  of 6.0 to 7.0 during the course of the fermentation.

Under these conditions the process was made semicontinuous by merely draining off the fermented liquor and adding fresh sterile mash. The organisms remain as a slimy scum on the limestones and serve to start the succeeding fermentation. The fermentation was complete in 40 to 60 hrs. and there seemed every reason to suppose that its course would not be markedly influenced by the size of the fermenting vessel. It was decided, therefore, to repeat the experiments on a larger scale.

### *Apparatus.*

The apparatus used was of copper, tin-lined, and consisted of two closed vessels of about 10 gals. capacity each. One, which was used as a sterilizer or cooker, was fitted with stirring apparatus and an interior coil which was connected both to the steam and cold water system. The fermenter itself was a similar vessel, but without the steam coil, and was filled with broken lumps of limestone about the size of an egg. It was connected to the cooker by means of a  $\frac{3}{4}$  in. pipe. The fermenter and connecting pipe were both connected with the steam line so that they could be sterilized.

### *Mash.*

A solution of beet molasses<sup>1</sup> was found the material most conveniently handled in the large size apparatus. The molasses used contained 1.05 g. of sugar (determined as dextrose by reduction after hydrolysis) per cc. For fermentation it was diluted to 15 times its volume with water. This mash therefore contained 70 mg. total sugar per cc.

### *Example of an Experiment.*

The fermenter and connecting pipe were heated under 10 lbs. steam pressure for 6 hrs. on three successive days and then allowed to cool to 40°, air being admitted through a cotton filter. 0.5 gal. of beet molasses was diluted to 7.5 gals. with water and heated in the cooker

<sup>1</sup> Information had been received at this time from the Department of Agriculture and also from the War Industries Board that large quantities of this molasses were available for fermentation; it was found later, however, that there was actually very little of the substance on the market.

under 15 lbs. pressure for 4 hrs. The mash was then cooled at 40° (by running water through the coil), forced into the fermenter with air pressure, and inoculated with 1 gal. of fermenting mash. This had been sterilized in a large Pasteur flask and inoculated 24 hrs. previously with 5 agar slants of the organism. The temperature of the room in which the apparatus was placed was maintained at 40°C.

Samples were withdrawn and analyzed at intervals. The acetone and alcohol were determined by the method described in the paper on the laboratory experiments. The reaction (*pH*) of the mash was determined roughly by brom-cresol-purple, phenol red, or phenolphthalein.

The results of the analyses are shown in Table II.

It will be seen that, after the first refilling of the tank the fermentations were complete in 50 to 60 hrs. with a yield of 8 to 8.5 per cent of the sugar as acetone and 20 to 21 per cent as alcohol. Calculated as volume per cent of the original molasses, the acetone is 9 to 10, and the alcohol 22 to 23 per cent of the original volume. The alcohol was identified, as described in the paper on the laboratory experiments, as ethyl alcohol containing probably some propyl and some butyl. The hydrogen ion concentration varies from about *pH* 8.0 at the beginning to *pH* 6.0 at the end. The optimum condition for fermentation is within this range.

The fermented mash from the foregoing experiments was distilled and finally fractionated in glass, yielding about 75 per cent of the calculated amount of acetone and alcohol in the form of the pure substances. As no special precaution was taken to make the fractionation strictly quantitative, the yield from the distillation agrees fairly well with the quantity found by analysis.

The experiments described above were repeated by Lieut. Ashe at the laboratory of Arthur D. Little, Inc., Cambridge, on a larger scale. In these experiments the fermenter held 160 to 175 gals. The fermentation was slightly slower than in the previous trials but gave a higher yield.

It had been found in the meantime that if the mash were brought to a *pH* of 8.5 to 9.5 by the addition of lime before fermentation the limestone in the fermenter could be replaced by brush or any similar inert material. The work was therefore repeated in Terre Haute

with a tank holding about 800 gals. and filled with brush instead of limestone chips. The fermentation took place in the same way and with the same yields as in the experiments described. In all the foregoing fermentations the principal difficulty encountered was the prevention of contamination by foreign organisms which destroyed the

TABLE II.

After Inoculation	ACETONE		ALCOHOL		Sugar per cc. of Solution	
	Per cc. of Solution	As per cent of Original Sugar	Per cc. of Solution	As per cent of Sugar		
Hrs.	Mg.	Per cent	Mg.	Per cent	Mg.	pH
0	0.07	0.1	...	...	70	8.5-9.0
24	0.24	0.3	...	...	..	6.5
48	2.0	3.0	...	...	..	6.0
72	3.7	5.3	8.4	12.0	11	6.0
Old mash run out and 5 gals. fresh run in						
1	0.4	0.5	...	...	65	8.0
48	5.4	7.7	...	...	10	6.0
50	5.6	8.0	14.5	20.0	8	6.0
Old mash run out and 5 gals. fresh sterile mash run in						
24	2.3	3.3	...	...	47	8.5
50	5.9	8.4	14.9	21.3	3	6.0
Old mash run out and 5 gals. fresh sterile mash run in						
24	2.8	4.0	...	...	40	8.3
52	5.8	8.3	14.0	20.0	4	6.0
Old mash run out and 5 gals. fresh mash run in						
24	3.0	...	...	...	..	8.1
48	5.0	...	...	...	..	...
56	5.9	...	...	...	..	...
72	6.0	8.5	15.2	21.7	3	6.0

acetone already formed, or interfered in other respects with the fermentation. The danger of contamination was particularly great when the old mash was drawn off. It was thought, therefore, that the process would be much easier to control as well as more rapid if the mash could be drawn off and run in in such a way as to avoid emptying the tank. This could be done by running in the fresh mash gently



at the bottom and allowing the fermented mash to flow off at the top. In this way the fermenter could be always kept filled and under pressure and the danger of contamination greatly lessened. It was found impossible to test this method in the laboratory as the fresh and fermented mash mixed too much in a small vessel. It was decided, therefore, to install an apparatus of about 1000 gals. capacity.

### *Apparatus.*

A steel tank 42 in. in diameter and 12 ft. high was set up and connected with the cooker and cooling coil as shown in Fig. 1. The various steam connections are apparent. It will be seen that the outlet of the tank was sealed off with steam which was always kept turned on slightly (Valve D). The tank was filled with broken corn cobs<sup>1</sup> and had four transverse perforated plates in order to prevent mixing.

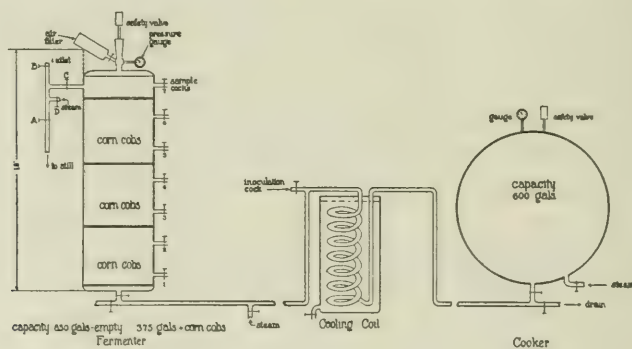


FIG. 1.

### *Mash.*

The following experiments were made, using poor grade cane molasses ("black strap") such as is used for fermentation. It contained 760 to 780 mg. of total sugar (as dextrose) per cc. It was found to be

<sup>1</sup> Corn cobs were used since they were the most readily available material and since they worked very well in laboratory experiments. They were found to be unsuited for use in a large tank as they gradually settled down and so greatly reduced the volume of liquid which the tank held. Coke or beech shavings, such as are used in vinegar towers, would probably be much more suitable.

slightly acid in reaction, so that it was necessary to use considerable lime to bring it to the proper degree of acidity. The sterilizing was complicated by the fact that the cooler was on a long (nearly 1000 ft.) steam line and therefore received very wet steam, so that the volume of mash increased greatly during sterilizing. This made it necessary to work with very dilute mashes since if high concentrations of molasses were used, so as to finish with the correct concentration, too much sugar was lost by caramelizing in the first part of the heating. Air-slaked lime was used to neutralize the molasses at the rate of 5 lbs. of lime per 5 gals. of molasses. Owing to the arrangement of the apparatus it was necessary to add this lime before sterilizing, although it was realized that this was not advisable. Nearly twice as much lime is needed if this is done and more sugar is destroyed. The lime should be pumped into the sterile mash after cooling.

### *Inoculation.*

This molasses was difficult to bring into fermentation and the difficulty was increased by the fact that no apparatus was available for "building up" a large inoculating culture. A series of six Pasteur flasks, each of 2 l. capacity, was therefore used, giving an inoculation of about 2 gals. This was not sufficient to start fermentation in the entire 400 gals. of mash, so that at first attempts were made to start 40 to 50 gals. of mash fermenting in the bottom of the tank and then to add the remainder of the mash slowly. It was not found possible to prevent contamination by this method, a butyric acid organism being the one which caused the most trouble. It was found possible, however, to keep the top of the tank (above Stopcock 1) under low steam pressure while the culture was growing in the lowest part of the tank. Fresh mash was then gradually added until the entire tank was filled. In this way all possibility of contamination was avoided.

The following fermentation was carried out in this manner:

*Sterilization.*

The fermenter was sterilized 10 hrs. a day for 8 days, under 20 lbs. steam pressure and then drained through the bottom cock. Cock 1 was now opened, all the others closed, and the steam valve at D opened slightly so as to allow a little steam to blow through the tank and out at Cock 1, Cocks A and B being closed.

*Mash.*

5 gals. of molasses, 120 gals. of water, and 5 lbs. of air-slaked lime were then sterilized under 15 lbs. pressure for 3 hrs., run through

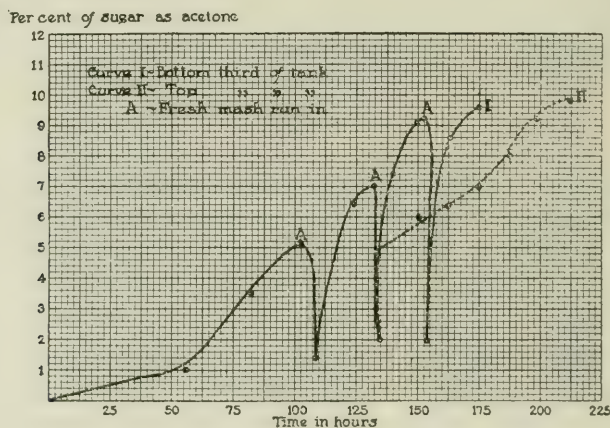


FIG. 2.

the cooling coil by steam pressure, and cooled to about 30°C. before entering the hot fermenter. The latter was filled to Stopcock 1; and four 2l. cultures run in through the inoculating cock and washed into the fermenter. These cultures had been inoculated from agar slants of *B. acetoethylicum*, and incubated for 24 hrs. The mash, owing to dilution while sterilizing, contained only about 9.5 mg. sugar per cc., whereas it was possible to ferment a mash containing more than three times as much sugar.

The pipe line connecting the cooler and fermenter was kept under 5 to 10 lbs. steam pressure when not in use and care was taken to prevent access of air to any part of the system. The outflow of gas

from the fermentation was regulated so as to maintain 2 to 5 lbs. pressure per sq. in. in the tank. This greatly reduces the danger of contamination.

TABLE III.

After inoculation	Volume Mash Run In	Sugar per cc. Fresh Mash	Mg. Acetone per 10 cc. at Cock No.							ACETONE			Alcohol as per cent of Original Sugar
			1	2	3	4	5	6	7	Volume Mash run out	Per 10 cc. Spent Mash	In Spent Mash as per cent of Original Sugar	
Hrs.	Gals.	Mg.								Gals.	Mg.		
23	100	9.4	...	...	...	...	...	...	...	...	...	...	...
56	...	...	1.4	0.4	0.35	0.4	...	...	...	...	...	...	...
57	75	9.0	...	...	...	...	...	...	...	...	...	...	...
82	...	...	4.3	2.8	3.3	2.4	1.2	...	...	...	...	...	...
105	...	...	5.0	5.2	3.5	1.3	...	...	...	...	...	...	...
106	75	10.0	...	...	...	...	...	...	...	...	...	...	...
107	...	...	1.3	1.7	1.9	2.4	2.4	3.6	...	...	...	...	...
124	...	...	6.6	6.2	3.6	4.2	4.0	4.2	...	...	...	...	...
130	...	...	7.4	6.6	4.5	4.0	4.0	4.4	...	...	...	...	...
131	75	9.6	...	...	...	...	...	...	...	50	4.3	4.3	...
131.5	...	...	1.7	2.2	3.2	4.8	5.0	5.0	5.0	...	...	...	...
139	...	...	7.4	7.6	5.6	5.2	5.4	5.4	5.4	...	...	...	...
147	...	...	9.2	9.0	7.4	6.2	6.0	6.0	6.2	...	...	...	...
152	...	...	10.0	8.6	7.2	6.4	6.2	5.8	6.0	...	...	...	...
152.5	100	9.5	...	...	...	...	...	...	...	100	6.0	6.0	...
153	...	...	1.8	2.0	2.6	3.6	4.0	5.8	5.4	...	...	...	...
164	...	...	9.0	8.0	8.8	7.2	7.0	6.4	6.3	...	...	...	...
164.5	50	9.8	...	...	...	...	...	...	...	50	6.4	6.4	...
175	...	...	8.8	9.4	10.4	7.4	7.0	7.0	7.0	...	...	...	...
175.5	50	9.7	...	...	...	...	...	...	...	50	7.0	7.0	...
187	...	...	9.6	9.2	9.2	8.0	8.0	8.0	8.0	...	...	...	...
188	50	9.65	...	...	...	...	...	...	...	50	8.0	8.0	20.0
199	...	...	10.6	8.9	8.9	9.1	9.0	9.3	9.2	...	...	...	...
199.5	50	10.0	...	...	...	...	...	...	...	50	9.0	9.0	22.3
211	50	9.7	...	...	...	...	...	...	...	50	8.9	8.9	22.4

After the tank had been filled the temperature was kept at about 40°C. by allowing a stream of water at approximately that temperature to flow over it.

It will be seen from Table III and Fig. 2 that the concentration of acetone gradually increased in the liquid in the upper part of the



tank (Stopcocks 5, 6, 7), until it reached about 0.9 mg. per cc., corresponding to 8 to 9 per cent by weight of the sugar originally present. It then remained constant at this point. These changes are shown graphically in Fig. 2. The analysis of the lower part shows a decided drop immediately after every additional charge of mash was run in. The concentration of acetone then increases quite rapidly until it reaches 9 to 10 mg. per 10 cc. This shows that there is no excessive mixing under these conditions even in a tank of the size used in this experiment. It is possible therefore to keep such a tank in continuous fermentation by adding fresh mash at the bottom and allowing the fermented mash to run off at the top. The larger and higher the tank the more rapidly the mash could be run through.

The reaction of the mash varied from  $pH$  8.0 to 9.0 in the fresh mash to  $pH$  6.0 to 7.0 in the spent mash. It was therefore always near the optimum for fermentation.

#### SUMMARY.

A brief description has been given of an organism which produces acetone and ethyl alcohol.

A method for conducting a continuous fermentation with molasses has been suggested and an experimental fermentation described.

## THE ISOMERIC HYDROXYPHENYLARSONIC ACIDS AND THE DIRECT ARSEINATION OF PHENOL.

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(Received June 30, 1919.)

The present study of the action of arsenic acid upon phenol has demonstrated that this reaction is more complicated than has been heretofore assumed. As we shall set forth below, not only is *p*-hydroxyphenylarsonic acid formed in the reaction, but several other related substances as well.

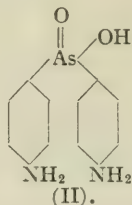
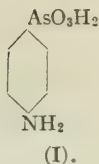
Ehrlich and Berthelm,<sup>1</sup> in establishing the nature of the substance now known as arsanilic acid or *p*-amino-phenylarsonic acid (I), obtained by heating aniline with arsenic acid, drew the analogy between this substance and sulfanilic acid, obtained by a parallel method. As far as has been determined in these reactions, the substituent enters only the position *para* to the amino group. It was then found that the method of direct arsenation was capable of extension to other simple aromatic amines with a free *para* position. Only in the case of a few *para*-substituted arylamines was it possible to force the arsonic acid into the *ortho* position and then only with great difficulty as evidenced by the very poor yields.

Benda,<sup>2</sup> and independently, Pyman and Reynolds<sup>3</sup> later found that this reaction did not stop at the formation of the primary arsonic acid but that this condensed further to an appreciable extent with a second molecule of the amine to form the secondary arsonic acid (II) in which the arsonic acid residue again entered the *para* position in the second arylamine nucleus.

<sup>1</sup> *Ber.*, **40**, 3292 (1907).

<sup>2</sup> *Ibid.*, **41**, 2367 (1908).

<sup>3</sup> *J. Chem. Soc.*, **93**, 1184 (1908).



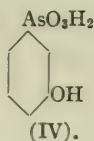
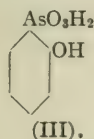
The method of direct arsenation has also been extended to include the simpler phenols. According to the method outlined in *D. R. P.* 205616, in which phenol, *o*- and *m*-cresol are heated with arsenic acid, substances are obtained in which the arsonic acid residue enters the position *para* to the phenolic group. Further than this the patent does not go; and, as far as we are aware, more recent work has applied the reaction only to the arsenation of resorcin and its mono-methyl ether.<sup>1</sup> From previous work, therefore, it would seem that the arsenation of phenolic compounds parallels that of the amines in that only *p*-hydroxy-arsonic acids have thus far been isolated.

In connection with the preparation of *p*-hydroxyphenylarsonic acid essentially according to the method outlined in *D. R. P.* 205616 we have had the opportunity of studying this reaction more fully. After isolating the *para* acid as the sodium salt, we were struck by the amount of material contained in the mother liquors which showed all the reactions of an aromatic arsonic acid and which obviously contained but relatively negligible amounts of the *p*-hydroxy acid. Furthermore, this mother liquor yielded an intense wine-red color with ferric chloride solution, whereas *p*-hydroxyphenylarsonic acid gives no such color test. It therefore seemed likely that we were dealing with isomeric hydroxy compounds which were also products of the reaction. Just as the arsenation of aniline has been compared with the sulfonation of this base, it seemed also logical to compare the arsenation of phenol with the sulfonation of this compound. As is well known, depending upon the conditions of sulfonation, varying proportions of both *o*- and *p*-phenolsulfonic acids may be obtained.

Accordingly, in order to learn the properties of both *o*- and *m*-hydroxyphenylarsonic acids (III) and (IV), neither of which had hith-

<sup>1</sup> Bauer, *Ber.*, **48**, 509 (1915).

erto been described, we prepared these compounds by diazotization of *o*- and *m*-arsanilic acids. Whereas phenol-*m*-arsonic acid resembled the *para* compound in giving no color test with ferric chloride, the phenol-*o*-arsonic acid gave the identical wine-red color observed with the mother liquors from phenol-*p*-arsonic acid.



It was then found possible to isolate a basic barium salt from these mother liquors which yielded a free acid identical with the hydroxy compound obtained from *o*-arsanilic acid. The amount of this substance isolated was approximately  $\frac{1}{4}$  of the yield of *p*-hydroxyphenylarsonic acid. We hope by varying the conditions of the arsenation to determine whether or not the small yield is due to the migration of the arsonic acid residue from the *o*- to the *p*-position at the elevated temperature employed, a process which has been shown to occur in the sulfonation of phenol. We have not succeeded in isolating a *meta* acid from the mother liquors and it seems hardly likely on theoretical grounds that one should be formed in the arsenation of phenol.

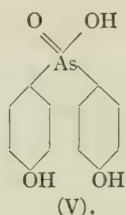
A comparison of the solubilities of the 3 phenolarsonic acids with those of the isomeric arsanilic acids proved interesting. Whereas in the latter series the *ortho* acid is most soluble and the *meta* compound the least soluble, with the former compounds the *o*-hydroxy acid unexpectedly proved to be the least soluble of the three. Thus it can be readily recrystallized from water without serious loss. There is little difference, on the other hand, between the solubilities of the *m*- and *p*-phenolarsonic acids.

We are continuing the study of the *o*- and *m*-hydroxyphenylarsonic acids and their reduction products, nitro derivatives, etc.

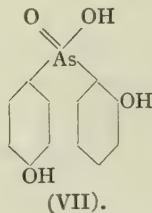
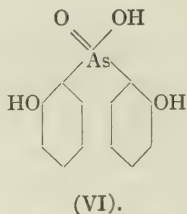
On careful acidification of the mother liquor from the barium salt of the phenol-*o*-arsonic acid we were able to isolate two other substances, both of which gave analytical figures for the secondary dihydroxydiphenylarsonic acids. These substances differed from one another in melting points and in the fact that in aqueous suspension one gave no



color with ferric chloride solution, while the other, lower melting substance, exhibited the same pronounced wine-red color so characteristic of phenol-*o*-arsonic acid. The higher melting acid possessed the same melting point as the *p,p'*-dihydroxy-diphenylarsonic acid (V), obtained according to Benda<sup>1</sup> by diazotization of *p,p'*-diamino-diphenylarsonic acid, and agreed in other properties with this substance. We therefore conclude that our first substance is identical with Benda's compound.



The color reaction exhibited by our second substance indicates that it is a secondary *o*-hydroxy-arsonic acid. There are two substances which might possibly be formed in the arsenation of phenol and exhibit the properties of the isolated compound; *o,o'*-dihydroxy-diphenylarsonic acid (VI), and *o,p'*-dihydroxy-diphenylarsonic acid (VII).



Since the secondary arsonic acids no doubt arise from the reaction of a second molecule of phenol with the primary hydroxyarsonic acids already formed, it is evident that VII could be formed in two ways; by the elimination of water between *o*-hydroxyphenylarsonic acid and the *para* hydrogen atom of a second phenol molecule, or by the condensation of *p*-hydroxyphenylarsonic acid with the *ortho* hydrogen of a second phenol molecule. VI, on the other hand, can only be formed from *o*-hydroxyphenylarsonic acid which is present

<sup>1</sup> *Loc. cit.*

only in minor amounts. Therefore, since the yield of our second substance was approximately equal to that of the *p,p'*-compound recovered, we are inclined to consider Formula VII as the more probable. We are at present attempting to establish the structure of the compound by other means. It also seems very likely that smaller amounts of VI are formed in the reaction and we are continuing the search for this substance.

The formation of the secondary acids extends the analogy to the sulfonation of phenol, since among the products of the latter is found dihydroxy-diphenylsulfone, and is likewise similar to the production of *p,p'*-diamino-diphenylarsonic acid in the arsenation of aniline. The formation of tertiary arsinoxides is likewise suggested, and we shall turn our attention to the possibility of isolating these among the products of the reaction. We shall also extend the study to the arsenation of other phenolic compounds, since similar products should result in the arsenation of the cresols, etc.

Finally, after the present work had long been under way, an article appeared by Conant<sup>1</sup> on the arsenation of phenol for the preparation of *p*-hydroxyphenylarsonic acid. It will be seen that the conditions employed by us for the interaction of phenol and arsenic acid, with a few exceptions, are similar to those used by him and to those outlined in *D. R. P.* 205616, which, however, specifies "crystalline arsenic acid." However, it is entirely unnecessary in working up the melt to be satisfied with an impure sodium salt such as is isolated by Conant's method. As will be found in the experimental part of the present communication, and as has already been claimed by Kay,<sup>2</sup> it is a simple matter to obtain satisfactory yields of pure sodium *p*-hydroxyphenylarsonate uncontaminated by other salts or by tarry by-products. As a matter of fact, the amount of tar formed in the method we have used is practically negligible and occasioned no difficulty. We are convinced that the direct arsenation of phenol is by far the simplest and most economical method for the preparation of this substance, so important in the manufacture of arsphenamine.

<sup>1</sup> THIS JOURNAL, **41**, 431 (1919).

<sup>2</sup> *Eng. Pat.* 6,322 (1915).

## EXPERIMENTAL.

(A) *o*- and *m*-Hydroxyphenylarsonic Acids.

*o*-Hydroxyphenylarsonic Acids.—This substance was isolated in the direct arsenation of phenol as described in Section B, and in order to prove its structure it was also synthesized directly from *o*-arsanilic acid.<sup>1</sup>

11 g. of *o*-arsanilic acid were dissolved in 100 cc. of *N* hydrochloric acid, chilled, and diazotized with 50 cc. of *N* sodium nitrite solution. On standing at room temperature a steady, copious evolution of nitrogen occurred which was completed after an hour by gentle heating. The mixture was boiled with bone black, and the colorless filtrate treated with 50 cc. of *N* sodium hydroxide solution. On concentrating on the water bath the new hydroxyphenylarsonic acid separated as a crust of long, stout needles. A few drops of dil. hydrochloric acid were added on cooling to insure proper acidity and the acid was filtered off and washed with ice water. The yield was 9.5 g. The acid is very easily soluble in hot water and forms supersaturated solutions on cooling, which rapidly crystallize when rubbed, yielding a mass of rosetts of minute, colorless needles. When crystallization occurs slowly, as on concentrating its aqueous solutions, the acid separates as crusts of long, lustrous needles. Contrary to its *p*-isomer, it is rather sparingly soluble in cold water. The solubility relationships, therefore, do not parallel those found in the comparison of *o*- and *p*-arsanilic acids, the latter being by far the less soluble.

*o*-Hydroxyphenylarsonic acid is readily distinguished from the *para* compound by the color which it gives with ferric chloride solution. Whereas the *p*-isomer gives no color with this reagent even very dilute solutions of the *ortho* acid give a pronounced wine red color. *m*-Hydroxyphenylarsonic acid, described below, resembles the *para* compound in giving no color with ferric chloride, so that this reaction may therefore be used to distinguish the *ortho* acid from its isomers.

When rapidly heated to 185°, then slowly, *o*-hydroxyphenylarsonic acid begins to soften at 185°, but is not completely melted until 196°

<sup>1</sup> THIS JOURNAL, 40, 1583 (1918).

is reached, then forming a liquid filled with bubbles. It is soluble in methyl or ethyl alcohol and but sparingly so in hot acetone or chloroform. Although difficultly soluble in glacial acetic acid, it dissolves readily on boiling. In alkaline solution, it couples readily with diazotized sulfanilic acid, yielding a bright orange solution. It is readily precipitated from concentrated solutions of its salts by hydrochloric acid, but redissolves on adding an excess. Acetic acid does not readily displace it from its salts.

Subs., 0.3273:  $\text{Mg}_2\text{As}_2\text{O}_7$ , 0.2298.

Calc. for  $\text{C}_6\text{H}_7\text{O}_4\text{As}$ : As, 34.36. Found: 33.87.

*Sodium Salt*.—A suspension of the acid in a small amount of hot water was neutralized to litmus with strong sodium hydroxide solution and then treated with an equal volume of alcohol. The sodium salt, which separated on rubbing, was recrystallized from 50% alcohol, separating on standing in the cold as glistening, hexagonal platelets which contain 4 molecules of water of crystallization when air-dry. The salt is readily soluble in water and gives no immediate precipitates with calcium or barium salts but in the latter case, on rendering alkaline to phenolphthalein, what is probably a basic barium salt separates as rosetts of colorless, microscopic needles. The heavy metals cause immediate flocculent precipitates.

Subs., air-dry, 0.7476: loss, 0.1713 *in vacuo* at  $100^\circ$  over  $\text{H}_2\text{SO}_4$ .

Calc. for  $\text{C}_6\text{H}_5\text{O}_4\text{AsNa} \cdot 4\text{H}_2\text{O}$ :  $\text{H}_2\text{O}$ , 23.07. Found: 22.92.

Subs., anhydrous, 0.2846:  $\text{Mg}_2\text{As}_2\text{O}_7$ , 0.1820.

Calc. for  $\text{C}_6\text{H}_5\text{O}_4\text{AsNa}$ : As, 31.20. Found: 30.87.

*m-Hydroxyphenylarsonic Acid*.—This substance was prepared as follows from *m*-arsanilic acid.<sup>1</sup> 11 g. of *m*-arsanilic acid were dissolved in 100 cc. of 10% sulfuric acid, chilled, and diazotized with a solution of 3.6 g. of sodium nitrite. The solution was diluted to 250 cc. and warmed to  $60^\circ$ , when a steady evolution of nitrogen occurred. When this had ceased, the sulfuric acid was completely removed with barium hydroxide solution. The filtrate was acidified with acetic acid, heated, and then treated with lead acetate solution as long as a precipitate formed. The lead salt separated at first in amorphous

<sup>1</sup> THIS JOURNAL, 40, 1583 (1918).



form, but rapidly changed to aggregates of microscopic platelets. After filtering off and washing, it was shaken with an excess of 10% sulfuric acid until completely decomposed. The filtrate from the lead sulfate was in turn treated with barium hydroxide solution until a test portion no longer showed the presence of dissolved barium and sulfate ions. The mixture was then treated with bone black and the clear, colorless filtrate boiled almost to dryness. The oily residue quickly crystallized on rubbing, giving 8 g. of the new arsonic acid. Recrystallized from a small volume of water and chilled to 0°, it separated as aggregates of rhombic crystals which melt slowly from 159° to 173°. It is readily soluble at room temperature in water, methyl and ethyl alcohols, and in boiling acetic acid. It is also somewhat soluble in hot acetone but practically insoluble in chloroform or benzene. It gives no color with ferric chloride solution and couples readily in alkaline solution with diazotized sulfanilic acid, giving a bright orange color.

Subs., 0.3045:  $\text{Mg}_2\text{As}_2\text{O}_7$ , 0.2135.

Calc. for  $\text{C}_6\text{H}_7\text{O}_4\text{As}$ : As, 34.36. Found: 33.84.

*Sodium Salt*.—A suspension of the acid in a very small amount of hot water was neutralized to litmus with sodium hydroxide solution. Since the salt could not be obtained by the addition of alcohol, it was allowed to evaporate and when almost dry, gradually crystallized in rosetts of flat needles. It was too soluble to be recrystallized and acetone precipitated the concentrated aqueous solution as a pasty mass. For analysis this was dried *in vacuo* at 100° over sulfuric acid.

Subs., 0.2854:  $\text{Mg}_2\text{As}_2\text{O}_7$ , 0.1833.

Calc. for  $\text{C}_6\text{H}_6\text{O}_4\text{AsNa}$ : As, 31.20. Found: 31.00.

A solution of the salt gives no precipitates with barium or calcium salts but insoluble precipitates with those of the heavy metals.

### (B) *The Arsenation of Phenol.*

*p*-Hydroxyphenylarsonic Acid.—480 g. of 80% aqueous arsenic acid were boiled in an open flask, allowing the water to escape until the temperature reached 150°. Then 200 g. of molten phenol were poured in at once and an air condenser attached, the flask being then

placed in an oil bath, the temperature of which was maintained at 155–60°. The contents of the flask boiled, rendering mechanical stirring unnecessary, and heating was continued for 7 hours. The air condenser acts as an efficient reflux avoiding undue loss of phenol. At the end the somewhat dark-colored liquid, which at first consisted of two layers, was homogeneous. It was diluted with about 2 liters of water, whereupon a small amount of tar precipitated, and, without filtering, a strong, hot solution of barium hydroxide was then added with vigorous stirring until the solution became just neutral to litmus, the unchanged arsenic acid being completely removed as the barium salt, which also efficiently collects the small amount of tar. It is important to avoid an unnecessary excess of barium hydroxide, since if the reaction becomes too strongly alkaline, the hydroxyphenylarsonic acids may also be precipitated. The clear, almost colorless filtrate from the copious barium arsenate precipitate was then treated with just enough sulfuric acid to remove the dissolved barium, a preliminary heating greatly facilitating the subsequent filtration of the barium sulfate. The precipitation is easily followed during the addition of the sulfuric acid by tests with filtered samples, and it is a simple matter to strike the point at which the filtrate no longer gives a test for either barium or sulfuric ion.

The filtrate was next concentrated to about a liter, preferably *in vacuo*, and then neutralized to litmus with sodium hydroxide. The solution of the salts was then concentrated further to small bulk, whereupon a partial crystallization of sodium *p*-hydroxyphenylarsonate occurred. The mixture was heated until this was redissolved, a very small amount of water added if necessary; and then treated while still hot with several volumes of alcohol until a slight permanent turbidity was reached. On rubbing, the sodium salt quickly crystallized out, and after several hours of thorough chilling it was filtered off and washed with 85% alcohol. The yield averages about 120 g. and the colorless product so obtained is a practically pure sodium *p*-hydroxyphenylarsonate giving no test for arsenate or sulfate ion and no color with ferric chloride solution. Hence it is seen that contamination with sodium sulfate, as occurs in Conant's method,<sup>1</sup> is an unnecessary complication.

<sup>1</sup> *Loc. cit.*

Subs., air-dry, 0.6264: loss, 0.0781 *in vacuo* at 80° over H<sub>2</sub>SO<sub>4</sub>.

Calc. for C<sub>6</sub>H<sub>6</sub>O<sub>4</sub>AsNa.2H<sub>2</sub>O: H<sub>2</sub>O, 13.04. Found: 12.46.

Subs., anhydrous, 0.2637: Mg<sub>2</sub>As<sub>2</sub>O<sub>7</sub>, 0.1708.

Calc. for C<sub>6</sub>H<sub>6</sub>O<sub>4</sub>AsNa: As, 31.20. Found: 31.26.

If it is desired to isolate the free *p*-hydroxyphenylarsonic acid, the filtrate from the barium sulfate is concentrated *in vacuo* without the addition of sodium hydroxide, a thick syrup of a mixture of free arsonic acids being obtained. On dissolving this in several volumes of hot glacial acetic acid, a faintly colored solution is obtained which gradually sets to a thick paste of colorless crystals of *p*-hydroxyphenylarsonic acid on chilling and rubbing. After standing 24 hours in the refrigerator it is filtered off and washed with small portions of cold glacial acetic acid. The yield of the arsonic acid, which melts at 170–3° with preliminary softening, averages 40 grams.

Subs., 0.2573: Mg<sub>2</sub>As<sub>2</sub>O<sub>7</sub>, 0.1850.

Calc. for C<sub>6</sub>H<sub>7</sub>O<sub>4</sub>As: As, 34.36. Found: 34.70.

Isolation of the substance as the sodium salt as first described is therefore preferable from the standpoint of yield. We have nothing to add to the properties of the acid and its sodium salt as already recorded in the literature.

*o*-Hydroxyphenylarsonic Acid.—The alcoholic mother liquor from the sodium salt of *p*-hydroxyphenylarsonic acid was concentrated to remove the alcohol and then diluted to about 750 cc. with water. A few drops of this solution gave the intense wine-red color characteristic of *o*-hydroxyphenylarsonic acid on treating with a drop of ferric chloride solution. The solution was treated with aqueous sodium hydroxide until strongly alkaline to phenolphthalein, and then with an excess of concentrated barium chloride solution. The barium salt of *o*-hydroxyphenylarsonic acid separated as a heavy powder on rubbing, and an additional small quantity was obtained by heating the filtrate from this to boiling. The combined fractions of the salt were suspended in water and treated with dil. sulfuric acid in slight excess and then the excess of sulfuric acid precipitated by the addition of just enough barium hydroxide solution so that the filtrate contained neither barium nor sulfate ions. The colorless filtrate was concentrated on the water bath to crystalliza-

tion, the *o*-hydroxyphenylarsonic acid separating as a characteristic crust of long, lustrous needles. After thorough chilling, it was filtered off and dried. The yield was 14 g. Recrystallized from hot water it formed flat, colorless needles which gave a wine-red color with ferric chloride solution, and proved identical in all respects with the substance obtained from *o*-arsanilic acid as described above. A mixture of the substances obtained by both methods showed no alteration in the melting point.

Subs., 0.3279:  $\text{Mg}_2\text{As}_2\text{O}_7$ , 0.2310.

Calc. for  $\text{C}_6\text{H}_6\text{O}_4\text{As}$ : As, 34.36. Found: 34.00.

For further identification, it was converted into the sodium salt, which separated from 50% alcohol as hexagonal, microscopic platelets containing 4 molecules of water of crystallization, just as in the case of the salt prepared from the sample synthesized from *o*-arsanilic acid.

Subs., 0.6372: loss, 0.1475 *in vacuo* at  $100^\circ$  over  $\text{H}_2\text{SO}_4$ .

Calc. for  $\text{C}_6\text{H}_6\text{O}_4\text{AsNa} \cdot 4\text{H}_2\text{O}$ :  $\text{H}_2\text{O}$ , 23.07. Found: 23.15.

Subs., anhydrous, 0.2508:  $\text{Mg}_2\text{As}_2\text{O}_7$ , 0.1635.

Calc. for  $\text{C}_6\text{H}_6\text{O}_4\text{AsNa}$ : As, 31.20. Found: 31.48.

*p,p'*-Dihydroxy-diphenylarsonic Acid.—The mother liquor from the barium salt of *o*-hydroxyphenylarsonic acid was treated with hydrochloric acid until only faintly alkaline and then concentrated to about one liter. The chilled solution was then further treated with 1:1 hydrochloric acid, causing the separation of an oily precipitate which partly dissolved on further addition of the acid until definitely acid to congo red. On rubbing, crystallization slowly started, and on continued manipulation the oily precipitate also slowly solidified. After 4 hours' standing, the deposit was filtered off and washed with water. An aqueous suspension gave a wine-red color with ferric chloride due to contamination with another secondary arsonic acid to be described below. On dissolving in hot 50% acetic acid and letting stand in the refrigerator, colorless, prismatic needles separated with a yield of 10 g., melting slowly with effervescence at  $250-1^\circ$  (uncor.). Benda<sup>1</sup> gives  $239^\circ$  as the melting point of *p,p'*-dihydroxy-diphenylarsonic acid obtained by diazotization of *p,p'*-diamino-diphenylarsonic

<sup>1</sup> Benda, *Ber.*, **41**, 2371 (1908).



acid. A sample of this substance prepared by us according to Benda also melted at  $250-1^{\circ}$  with effervescence, as did a mixture of this substance and that isolated as described above. Other properties were also identical. An aqueous suspension of the pure substance gave no color test with ferric chloride solution. It is practically insoluble in cold water, but dissolves appreciably on boiling and separates on cooling as short, colorless, prismatic needles. It is readily soluble in hot 50% or 95% alcohol or glacial acetic acid and in cold methyl alcohol, but very sparingly so in ether and acetone. Benzene and chloroform do not dissolve the substance. It is readily soluble in alkali and carbonate solutions and in 10% hydrochloric acid. In alkaline solution, it couples with diazotized sulfanilic acid, yielding an orange solution. There is no doubt that this substance is identical with that described by Benda as dihydroxy-diphenylarsonic acid.

Subs., 0.2573:  $\text{Mg}_2\text{As}_2\text{O}_7$ , 0.1348.

Calc. for  $\text{C}_{12}\text{H}_{11}\text{O}_4\text{As}$ : As, 25.49. Found: 25.30.

*o,p'(?)-Dihydroxy-diphenylarsonic Acid*.—On standing several hours longer, the aqueous mother liquor from the *p,p'*-arsonic acid gradually deposited heavy aggregates of stout, glistening prisms which increased on rubbing. After about 48 hours, these were filtered off and washed with water. Four g. were obtained, melting at  $215-6^{\circ}$  with effervescence and giving a deep wine-red color with ferric chloride solution. The mother liquor was treated with sodium acetate solution until neutral to congo red, causing the precipitation of a small amount of resinous material. On standing, crystallization again started and was facilitated by rubbing. An additional 4 g. of material were thus obtained which also melted at  $215-6^{\circ}$  with effervescence and gave a deep wine-red color with ferric chloride solution. The combined fractions were dissolved in a small volume of hot 50% acetic acid and separated slowly on cooling as short, stout, glistening prisms which melted at  $215-7^{\circ}$  (uncor.) to a liquid filled with bubbles. Repeated recrystallization did not alter the melting point. A further small quantity of this substance was obtained on concentrating the 50% acetic acid mother liquor obtained in the recrystallization of the previously described *p,p'*-dihydroxy-diphenylarsonic

acid and fractional recrystallization of the product so obtained. The compound is appreciably soluble in boiling water and separates on cooling as flat, glistening, prismatic needles. It is somewhat soluble in 50% or 95% alcohol, but quite readily so on boiling. It is also soluble in hot acetic acid and cold methyl alcohol, very sparingly in acetone and ether, and insoluble in benzene and chloroform. It dissolves readily in alkali and carbonate solutions and in 10% hydrochloric acid. In alkaline solution it couples with diazotized sulfanilic acid to yield an orange solution.

Subs., 0.2508:  $\text{Mg}_2\text{As}_2\text{O}_7$ , 0.1322.

Calc. for  $\text{C}_{12}\text{H}_{11}\text{O}_4\text{As}$ : As, 25.49. Found: 25.43.

The substance is readily distinguished from the *p,p'*-isomer by the fact that in aqueous suspension it gives an intense wine-red color with ferric chloride solution, similarly to *o*-hydroxyphenylarsonic acid. We, therefore, conclude that it is either *o,p'*-dihydroxy-diphenylarsonic acid or the *o,o'*-isomer. Since *o*-hydroxyphenylarsonic acid is now definitely established as a product of the arsenation of phenol, it seems likely that this substance may react with a second molecule of phenol to form the secondary arsonic acid, the arsonic residue entering the position *para* to the hydroxyl groups just as the *p,p'*-hydroxy compound results from the further alteration of *p*-hydroxyphenylarsonic acid. The yield of the acid also makes it seem probable that we are dealing with the *o,p'*-dihydroxy compound rather than the *o,o'*-acid, as well as the additional possibility of its formation by condensation of *p*-hydroxyphenylarsonic acid with a further molecule of phenol in the *o*-position. It is also probable that the *o,o'*-dihydroxy acid may occur among the reaction products but in such small amount as to render its isolation difficult. We are at present attempting to establish with certainty the identity of the new compound by synthetic means.



## CERTAIN AMINO AND ACYLAMINO PHENOL ETHERS.

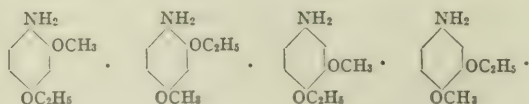
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In the course of recent chemotherapeutic studies it was found necessary to prepare as intermediates certain aromatic amines containing hydroxy, methoxy, and ethoxy groups, as well as multiples and combinations of these. While the monosubstituted compounds of this type have been adequately studied there are many gaps in the series of disubstituted amines containing the groups in question, as well as a number of inaccuracies in the descriptions of compounds which have already been prepared. In the present paper we have endeavored to fill some of these gaps and correct those inaccuracies which we have encountered, confining the discussion to certain amines and acylamino derivatives of anisole and phenetole and of the simpler mono- and diethers of pyrocatechol and resorcinol.

Of primary interest are perhaps the 4 isomeric methoxy-ethoxy-anilines:



M. P.				
The base.....	27.5-8.5°	22.5°	55°	81.5-2°
Acetamino deriv.....	117.5-8.5	100.5-1	148.5-50	145-6
Chloroacetyl amino deriv.	97.5-8	126-7	133-4	135.5-6

Of these the two derived from resorcinol have never been prepared, as far as we have been able to find, while the two amino-pyrocatechol ethers apparently were isolated as their hydrochlorides by Wisinger,<sup>1</sup> who also prepared the acetamino compounds and states that the free bases are easily oxidizable oils. Wisinger prepared the hydrochlorides and the acetyl derivatives by reduction of the two isomers obtained by nitrating pyrocatechol methylethyl ether, and designated the

<sup>1</sup> *Monatsh.*, **21**, 1013 (1900).



isomers by the letters  $\alpha$ - and  $\beta$ -, being unable to determine which was the 3,4-methoxy-ethoxy- and which the 4,5-isomer. However, by ethylating 5-nitroguaiacol Paul<sup>1</sup> obtained a nitro ether corresponding to Wisinger's  $\beta$ -compound, whose  $\beta$ -methoxy-ethoxy-acetanilide would therefore be the 4,5-isomer, although the melting point given is considerably lower than that found by us for this compound. We have, on the other hand, found all four bases to be easily crystallizable and quite stable under ordinary conditions.

It seems also that 3-methoxy-4-ethoxy-acetanilide was prepared by Freyss<sup>2</sup> by ethylation of a "*p*-nitroguaiacol" (m. p. 104°), followed by reduction and isolation of the amine as the acetyl derivative. Freyss showed that the nitro group in his *p*-nitroguaiacol was in the position *para* to the hydroxyl group, so that his nitro ethyl ether should have corresponded to Wisinger's  $\alpha$ -compound. It melted 20° higher, however, so that Wisinger's product would seem to have been either an *ortho* nitro derivative or a mixture, a conclusion borne out by the low melting point obtained by Wisinger for the acetamino compound as well. In any event, the methods employed in the present paper were such as to render it certain that no confusion between the isomers could arise.

Regarding the methods used, the substances containing the *p*-amino-phenol grouping were prepared by reduction of the corresponding *p*-sulfo-phenylazo dye in ammoniacal solution by means of hydrogen sulfide, a method which we had used to good effect in the preparation of 4-amino-guaiacol.<sup>3</sup> Chloroacetyl derivatives were made according to the method devised by us and used in numerous instances already published.<sup>4</sup> All melting points above 140° are corrected to the short-stem thermometer basis.

#### EXPERIMENTAL.

##### (A) *Derivatives of the Ethers of Phenol, o- and m-Cresol.*

*Chloroacetyl-o-anisidine*,  $o\text{-CH}_3\text{OC}_6\text{H}_4\text{NHCOCH}_2\text{Cl}$ .—This substance may be obtained in almost quantitative yield from the base and

<sup>1</sup> *Ber.*, **39**, 2777 (1906).

<sup>2</sup> Freyss, *Zentr.*, 1901, I, 739.

<sup>3</sup> *THIS JOURNAL*, **41**, 467 (1919).

<sup>4</sup> *Ibid.*, **39**, 1439 (1917) and subsequent papers.

chloroacetyl chloride in dil. acetic acid solution in the presence of sodium acetate.<sup>1</sup> After recrystallization first from ligroin and then from absolute alcohol the melting point was 48.5–9° (corr.) with slight preliminary softening, thus confirming our original observation,<sup>2</sup> rather than that of Beckurts and Frerichs (51°).<sup>3</sup>

*Chloroacetyl-m-anisidine*.—The *m*-anisidine used in the preparation of this substance was prepared essentially as given by Reverdin and de Luc,<sup>4</sup> except that hydrolysis of the acetyl derivative was accomplished by boiling for  $\frac{1}{2}$  hour with approximately 5 parts of 1:1 hydrochloric acid. Practically all of the base boiled at 131° under a pressure of 14 mm.

Six g. of *m*-anisidine were chloroacetylated in the usual way. The solution remained clear, but on chilling and rubbing in a freezing mixture it soon set to a solid cake. After adding 2 volumes of water the chloroacetyl compound was filtered off, dried, and recrystallized by dissolving in hot benzene, treating with  $\frac{1}{2}$  volume of ligroin, and seeding. 6.9 g. separated as tufts of flat needles and long plates. Recrystallized again from absolute alcohol with the aid of a freezing mixture a portion melted constantly at 90.5–1.5° (corr.) with preliminary softening at 90°. It dissolves readily in the usual solvents with the exception of water and ligroin.

Subs., 0.3284: (Kjeldahl), 16.7 cc. 0.1 *N* HCl.

Calc. for  $C_9H_{10}O_2NCl$ : N, 7.02. Found: 7.12.

*Chloroacetyl-o-phenetidine*,  $o-C_2H_5OC_6H_4NHCOCH_2Cl$ .—This substance was obtained in almost quantitative yield, separating as an oil on dilution of the reaction mixture with an equal volume of water and soon crystallizing. A portion was recrystallized twice from 85% alcohol, separating as hexagonal rhombs melting at 65.5–7.0° (corr.) with slight, preliminary softening. It is less soluble in alcohol and ligroin than in the other usual organic solvents.

Subs., 0.3200: (Kjeldahl), 14.7 cc. 0.1 *N* HCl.

Calc. for  $C_{10}H_{12}O_2NCl$ : N, 6.56. Found: 6.44

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<sup>1</sup> *Loc. cit.*

<sup>2</sup> *J. Biol. Chem.*, **21**, 135 (1915).

<sup>3</sup> *Arch. Pharm.*, **253**, 233 (1915).

<sup>4</sup> *Ber.*, **47**, 1537 (1914).

*Chloroacetyl-m-phenetidine*.—The *m*-phenetidine used for the preparation of this substance was prepared from *m*-acetaminophenol and diethyl sulfate in the presence of alkali at 50–60°, but the yield was not as good as that reported by Reverdin and Lokietek<sup>1</sup> using ethyl bromide. Practically all of the base boiled at 144.5° under a pressure of 20 mm.

The chloroacetyl derivative was obtained in almost quantitative yield as in the preceding cases, separating from the reaction mixture at once and forming so thick a paste that the addition of more 50% acetic acid was found necessary in order to maintain fluidity. After diluting with water a portion of the collected solid was recrystallized from 85% alcohol, then from toluene, forming flat, glistening needles, melting at 125.5–6.5°. The compound is readily soluble in acetone or chloroform, somewhat less easily in alcohol, ether, or benzene at room temperature. It dissolves sparingly in cold toluene, easily on boiling, and is difficultly soluble in boiling water.

Subs., 0.2000: (Kjeldahl), 9.55 cc. 0.1 *N* HCl.

Calc. for  $C_{10}H_{12}O_2NCl$ : N, 6.56. Found: 6.69.

*Chloroacetyl-p-phenetidine*.<sup>2</sup>—This substance was also prepared in good yield as in the above cases.

*3-Methyl-4-methoxy-acetanilide*,  $3,4-CH_3(CH_3O)C_6H_3NHCOCH_3$ .—Nineteen g. of *p*-amino-*o*-cresol<sup>3</sup> were dissolved in 170 cc. of *N* hydrochloric acid and the solution treated with 24.5 g. of acetic anhydride, followed immediately by 100 cc. of saturated sodium acetate solution. The mixture was shaken vigorously for 10 minutes and the precipitate of *p*-acetamino-*o*-cresol then filtered off and washed with water. The yield was 20 g. The crude product was converted into the methyl ether by dissolving in 1.1 equivalents of *N* potassium hydroxide solution and shaking with one equivalent of dimethyl sulfate, filtering off the ether and repeating the process with the filtrate with  $\frac{1}{4}$  of the original amounts of alkali and dimethyl sulfate. The fractions were combined and a portion recrystallized from 50% alcohol, separating as large, nacreous, hexagonal scales which melted

<sup>1</sup> *Bull. soc. chim.*, [4] 17, 407 (1915).

<sup>2</sup> Bistrzycki and Ulfers, *Ber.*, 31, 2790 (1898); *Ger. pats.* 79,174; 84,654.

<sup>3</sup> Prepared according to *THIS JOURNAL*, 39, 2198 (1917).

at 103–3.5° with slight preliminary softening. The compound is readily soluble in alcohol, acetone, chloroform, or ether, and dissolves sparingly in cold benzene, easily on boiling. It also dissolves with difficulty in cold water, more easily on boiling, the undissolved portions melting to an oil.

Subs., 0.1714: (Kjeldahl), 9.6 cc. 0.1 *N* HCl.

Calc. for  $C_{10}H_{13}O_2N$ : N, 7.83. Found: 7.85.

*3-Methyl-4-methoxy-aniline*.—The crude acetamino ether was boiled for one-half hour with 5 parts of 1:1 hydrochloric acid. The solution was diluted with water, chilled, and made strongly alkaline with sodium hydroxide. The 3-methyl-4-methoxy-aniline immediately separated in crystalline form and was filtered off, washed with water, and recrystallized from 50% alcohol. The yield obtained as outlined above from 30 g. of *p*-acetamino-*o*-cresol was 13 g., an additional 1.4 g. being obtained by dilution of the mother liquors of the recrystallization. The melting point was 59–9.5°, as given by Bamberger and de Werra,<sup>1</sup> who obtained the base by decomposition of *m*-tolyl-hydroxylamine with methyl alcohol containing sulfuric acid. The amine is easily soluble in alcohol, acetone, benzene, or ether, and dissolves very sparingly in cold water, readily on boiling, separating as an oil which crystallizes on rubbing. It may also be obtained from hot ligroin as thick, almost colorless, hexagonal plates. An aqueous suspension slowly gives a deep blue-violet color with ferric chloride, and the base is readily diazotized, coupling with R-salt to give a deep red color.

*3-Methyl-4-methoxy-chloroacetanilide*.—The base was chloroacetylated as in the previous experiments, giving an excellent yield of the acetyl derivative on diluting the reaction mixture with a little water and rubbing. A portion was recrystallized twice from 60% alcohol, then by dissolving in warm benzene and adding an equal volume of ligroin. It forms delicate needles melting constantly at 90–2° with preliminary softening, and dissolving readily in the usual organic solvents except ligroin.

Subs., 0.1524: (Kjeldahl), 7.15 cc. 0.1 *N* HCl.

Calc. for  $C_{10}H_{12}O_2NCl$ : N, 6.56. Found: 6.57.

<sup>1</sup> *Ann.*, **390**, 175 (footnote) (1912).



*2-Methyl-4-methoxy-aniline*,  $2,4\text{-CH}_3(\text{CH}_3\text{O})\text{C}_6\text{H}_3\text{NH}_2$ .—Twenty g. of *p*-acetamino-*m*-cresol<sup>1</sup> were methylated as in the case of the *o*-compound. The yield of the acetamino ether was 14.5 g., agreeing in its properties with those recorded by Blangey,<sup>2</sup> who obtained the substance by acetylation of the amino compound. Thirteen g. of the acetamino ether were boiled for one hour with 65 cc. of 1:1 hydrochloric acid, diluted, chilled, and the solution was made strongly alkaline with 25% sodium hydroxide solution. The oily base was extracted with ether, and after drying over sodium sulfate the solvent was distilled off and the residue fractionated *in vacuo*. Nine g. of 2-methyl-4-methoxy-aniline were obtained, boiling at 144–52° (almost all at 146–7°) under a pressure of 23 mm. The almost colorless liquid crystallized as diamond-shaped platelets on chilling. These melted at 13–14° (corr.), the melting point being unchanged on recrystallization from ligroin. Bamberger and Blangey,<sup>3</sup> who obtained the base by treating *o*-tolyl-hydroxylamine with methyl alcohol containing sulfuric acid, give the melting point as 29–30°. In other respects their description was confirmed.

Subs., 0.1459: (Kjeldahl), 10.65 cc. 0.1 *N* HCl.

Calc. for  $\text{C}_8\text{H}_{11}\text{ON}$ : N, 10.22. Found: 10.23.

*2-Methyl-4-methoxy-chloroacetanilide*.—On treating 8.7 g. of 2-methyl-4-methoxy-aniline with a mixture of 45 cc. of glacial acetic acid and 45 cc. of saturated sodium acetate solution what appeared to be the acetate of the base crystallized at once. A clear solution was obtained, however, after adding an additional 110 cc. of 50% acetic acid. The solution was then chilled and treated cautiously with 7.2 cc. of chloroacetyl chloride, with continued cooling and vigorous shaking. Precipitation of the chloroacetyl derivative was completed by adding an equal volume of water. After recrystallization from 85% alcohol the yield was 11.5 g. Recrystallized again from toluene, in which the substance is very easily soluble at the boiling point and very sparingly so in the cold, it separates as hair-like needles which melt constantly at 134.5–5.5°. It is practically insol-

<sup>1</sup> THIS JOURNAL, 39, 2200 (1917).

<sup>2</sup> Dissertation, Zürich, 1903.

<sup>3</sup> *Ibid.*, also *Ann.*, 390, 174 (footnote) (1912).

uble in cold water but dissolves appreciably on boiling. It is sparingly soluble in cold alcohol, easily on boiling, and dissolves readily in acetone or chloroform.

Subs., 0.1749: (Kjeldahl), 8.15 cc. 0.1 *N* HCl.

Calc. for  $C_{10}H_{12}O_2NCl$ : N, 6.56. Found: 6.53.

*m*-Nitro-*p*-anisidine (3-nitro-4-methoxy-aniline).—This substance was prepared by nitrating acet-*p*-anisidide, according to German patent 101,778 and saponifying by boiling the nitro derivative with 25% sulfuric acid for  $\frac{1}{2}$  hour. The resulting solution was diluted with water, cooled somewhat, and made strongly alkaline. The base was extracted with ether, the solvent distilled off, and the residue dried on a porous plate. The crude product was dissolved in cold benzene and recrystallized by cautiously adding ligroin and rubbing. 16 g. of acet-*p*-anisidide gave 10.4 g. of the nitro-anisidine, melting at 55–7° and not at 50° as stated in the patent. Recrystallized twice from toluene, cooled, and treated with bone black the first time to remove a slight turbidity, the compound separated slowly as a hard crust of red prisms which melted constantly at 57–7.5° (corr.). As so obtained, the substance still contained a small amount of an amorphous residue which could be removed by dissolving in hot ether, filtering from the residue, adding ligroin, and letting the ether evaporate gradually. The nitro-anisidine gradually separated as orange-red prisms and plates which melted as above. It is readily soluble in the cold in acetone, alcohol, or ether, rather less so in benzene, and difficultly in cold toluene or water, readily on boiling. It dissolves in warm 10% hydrochloric acid with a pale yellow color, the hydrochloride separating on cooling as almost colorless, glistening platelets. It is readily diazotized, giving a sparingly soluble, orange-red dye with R-salt.

Subs., 0.1182: 16.6 cc. N (19.5°, 760 mm.).

Calc. for  $C_7H_8O_3N_2$ : N, 16.67. Found: 16.40.

3-Nitro-4-methoxy-chloroacetanilide.—Five g. of the nitro-anisidine were dissolved in 25 cc. of glacial acetic acid and chloroacetylated in the usual way after adding 25 cc. of saturated sodium acetate solution. The acyl derivative separated during the reaction and was filtered off

after adding several volumes of water. The yield was 6.7 g. A portion was recrystallized from alcohol, then twice from ethyl acetate, in which it is rather difficultly soluble, forming golden yellow, flat needles melting at 149.5–51.5°. The substance dissolves quite readily in cold acetone and is somewhat soluble in cold chloroform, more easily on boiling. It is very difficultly soluble in cold water but dissolves appreciably on boiling.

Subs., 0.1284: 12.5 cc. N (19.5°, 766 mm.).

Calc. for  $C_9H_9O_4N_2Cl$ : N, 11.46. Found: 11.46.

*3-Acetamino-6-methoxy-benzenesulfonic Acid*.—Thirty g. of 3-amino-6-methoxy-benzenesulfonic acid (prepared by sulfonating *p*-anisidine<sup>1</sup>) were pulverized, dissolved in 147 cc. of *N* sodium hydroxide solution, and shaken vigorously for 10 minutes with 18.2 cc. of acetic anhydride. The solution was then concentrated to dryness *in vacuo* and the residue taken up with acetone, filtered off, washed with acetone, and dried.<sup>2</sup> The yield of crude sodium 3-acetamino-6-methoxy-benzenesulfonate was 41.3 g.

A portion of the salt was dissolved in 2 parts of hot water and the solution treated with bone black and filtered. On adding to the filtrate an equal volume of 10% hydrochloric acid and rubbing the acid separated quickly as microscopic rhombs. These were filtered off, washed with a little 10% hydrochloric acid, and recrystallized from a small volume of water containing a few drops of acetic acid. The acid separated on cooling and seeding as minute, flat needles. When rapidly heated to 195°, then slowly, it intumesces at 197–8°, resolidifies, gradually turns yellow as the temperature is further raised, and finally melts again with gas evolution at about 250°. It is readily soluble in water and only sparingly in boiling methyl alcohol, ethyl alcohol, or acetic acid.

Subs., 0.1672: (Kjeldahl), 9.7 cc. 0.1 *N* HCl.

Calc. for  $C_9H_{11}O_6NS$ : N, 5.71. Found: 5.80.

*3-Acetamino-6-methoxy-benzene-sulfonamide*, 3,6- $CH_3CONH(CH_3O)-C_6H_3SO_2NH_2$ .—21.7 g. of the crude, dry sodium acetamino-methoxy-

<sup>1</sup> Bauer, *Ber.*, **42**, 2110 (1909).

<sup>2</sup> Cf. *THIS JOURNAL*, **39**, 2428 (1917).

benzenesulfonate were intimately mixed in a mortar with 16.8 g. of phosphorus pentachloride, transferred to a flask with a drying tube attached, and heated for one hour at 50°. Hydrogen chloride was copiously evolved and at the end the flask was evacuated for about  $\frac{1}{2}$  hour in order to remove as much of the phosphorus oxychloride as possible. The residue was then ground up with ice, filtered off when pulverulent, and the crude chloride allowed to stand for 2 days with 130 cc. of 1:1 ammonium hydroxide. Nine g. of crude amide were obtained in this way, and the product was sufficiently pure for conversion into the amino amide. Analysis showed that recrystallization from alcohol was insufficient to insure a pure product. As obtained in this way the sulfonamide forms faintly yellow, radiating aggregates of minute, compact crystals which are only difficultly soluble in boiling alcohol. When rapidly heated to 230°, then slowly, the substance melts to a dark liquid at 233–5.5°, with slow gas evolution.

Subs., 0.2510: (Kjeldahl), 19.35 cc. 0.1 *N* HCl.

Calc. for  $C_9H_{12}O_4N_2S$ : N, 11.48. Found: 10.80.

*3-Amino-6-methoxy-benzene-sulfonamide*.—6.5 g. of the crude acet-amino amide were boiled for  $\frac{1}{2}$  hour with 35 cc. of 1:1 hydrochloric acid. After cooling the solution was made just alkaline with ammonia and the precipitated amino-sulfonamide recrystallized from 50% alcohol. The yield was 3.4 g. Recrystallized again from 50% alcohol it separated from the cold, supersaturated solution as radiating, branched aggregates of minute, cream-colored spindles which melted at 184.5–6.0° with preliminary softening. It is difficultly soluble in the usual neutral organic solvents but dissolves readily in boiling water or 50% alcohol, less easily in boiling 95% alcohol. It dissolves in dil. hydrochloric acid or sodium hydroxide solution, the acid solution diazotizing readily and coupling with R-salt to give a deep red color. An aqueous solution gives a slowly developing brownish pink color with ferric chloride.

Subs., 0.1532: (Kjeldahl), 15.05 cc. 0.1 *N* HCl.

Calc. for  $C_7H_{10}O_3N_2S$ : N, 13.87. Found: 13.77.



(B) *Derivatives of the Ethers of 4-Amino-pyrocatechol.*

*3,4-Methylenedioxy-chloroacetanilide*,  $3,4\text{-CH}_2\text{O}_2\text{C}_6\text{H}_3\text{NHCOCH}_2\text{Cl}$ .—Four g. of 3,4-methylenedioxy-aniline hydrochloride (from the nitro compound with tin and hydrochloric acid in dilute alcohol) were dissolved in 40 cc. of 50% acetic acid and 20 cc. of saturated sodium acetate solution, chilled in ice-water, and treated with 3 cc. of chloroacetyl chloride. The resulting mixture was ground up in a mortar, diluted with an equal volume of water, and the precipitate filtered off and washed with water. After recrystallization from 85% alcohol the yield was 3.5 g., the substance forming both prismatic needles and glistening platelets. After 3 subsequent recrystallizations from toluene in which it is easily soluble at the boiling point, but difficultly in the cold, the substance separated entirely as plumes of microscopic needles which melted constantly at  $157.5\text{--}8.5^\circ$  with preliminary softening. It is readily soluble in acetone, somewhat less easily in chloroform, and only sparingly in cold alcohol, readily, however, on warming. It also dissolves in boiling water and only very difficultly in the cold. The compound gives a pale yellow color with sulfuric acid.

Subs., 0.1503: (Kjeldahl), 7.0 cc. 0.1 *N* HCl.

Calc. for  $\text{C}_9\text{H}_8\text{O}_3\text{NCl}$ : N, 6.56. Found: 6.52.

*4-Chloroacetyl-amino-guaiacol*,  $3,4\text{-CH}_3\text{O}(\text{HO})\text{C}_6\text{H}_3\text{NHCOCH}_2\text{Cl}$ .—16.7 g. of 4-amino-guaiacol<sup>1</sup> were dissolved in a warm mixture of 100 cc. of acetic acid and 100 cc. of saturated sodium acetate solution, rapidly chilled, and cautiously treated with 15 cc. of chloroacetyl chloride. The resulting solution was concentrated to small bulk *in vacuo* and the crude, crystalline chloroacetyl derivative recrystallized from a small volume of 50% alcohol. The yield was 17.5 g. Recrystallized from water with the aid of bone black, it forms slightly pinkish, thin, nacreous plates which melt at  $113\text{--}4^\circ$  with slight preliminary softening. The substance dissolves readily in acetone, alcohol, or hot water, less easily in hot benzene, and only sparingly in cold water or cold benzene. An aqueous solution gives a yellow-brown color with ferric chloride.

<sup>1</sup> THIS JOURNAL, 41, 467 (1919).

Subs., 0.1554: 8.8 cc. N (28.5°, 755 mm.).

Calc. for  $C_9H_{10}O_3NCl$ : N, 6.50. Found: 6.37.

5-Chloroacetyl-amino-guaiacol, 3,4- $HO(CH_3O)C_6H_3NHCOCH_2Cl$ .—5-Nitroguaiacol (m. p. 104°) was reduced according to Mameli.<sup>1</sup> After concentrating the detinned solution *in vacuo* and washing with a little 1:1 hydrochloric acid, the 5-amino-guaiacol hydrochloride (N, 7.83; calc., 7.98) formed practically colorless crystals which melted to a semifluid mass at about 160° and became completely fluid and decomposed at about 180° when rapidly heated. An aqueous solution gives a deep brown-red color with ferric chloride.

Six g. of the hydrochloride were dissolved in 25 cc. of water and chloroacetylated in the usual way after adding 10 cc. of acetic acid and 30 cc. of saturated sodium acetate solution. The substance crystallized on shaking and rubbing and was recrystallized from water containing a few drops of acetic acid, forming slightly pinkish hexagonal plates and prisms melting at 128–48° with preliminary softening. The yield was only 2.7 g., owing to considerable loss during the recrystallization. Two subsequent recrystallizations from toluene gave the melting point 140–50°, with preliminary softening, the compound forming pale pink, nacreous platelets. It dissolves in alcohol, ethyl acetate, boiling toluene, or boiling water, and is almost insoluble in cold toluene or benzene.

Subs., 0.1512: (Kjeldahl), 6.9 cc. 0.1 N HCl.

Calc. for  $C_9H_{10}O_3NCl$ : N, 6.50. Found: 6.39.

*p*-Sulfo-phenylazo-*o*'-ethoxy-phenol, *p*- $HO_3SC_6H_4N:NC_6H_3(OC_2H_5)-OH(3',4'-)$ .—23.1 g. of air-dry sodium sulfanilate (0.1 mol.) were dissolved in 400 cc. of ice and water, 7 g. of sodium nitrite added, and 60 cc. of 1:1 hydrochloric acid run in, with stirring. The resulting mixture was slowly added to a solution of 13.8 g. of *o*-ethoxy-phenol (guethol), keeping the temperature at 20° by adding ice.<sup>2</sup> After stirring for 15 minutes, the deep orange-brown solution was acidified strongly with conc. hydrochloric acid. On rubbing and stirring the dye separated quickly as glistening, coppery aggregates of platelets.

<sup>1</sup> *Chem. Centr.*, 1908, I, 25.

<sup>2</sup> Cf. the analogous preparation of 4-aminoguaiacol, *Loc. cit.*

After letting stand overnight in the ice box the dye was filtered off and washed with 10% hydrochloric acid and then with acetone. The yield was 26.3 g. A portion was recrystallized from water, in which it is difficultly soluble in the cold, easily on boiling, forming dark red platelets with a purple reflex. After washing with a little water, then with acetone, and air-drying, the substance contained approximately 2 molecules of water of crystallization. When rapidly heated the anhydrous compound melts and evolves gas at about  $220^{\circ}$ , with preliminary decomposition, while if the heating is slow above  $200^{\circ}$  it merely softens and gradually decomposes as the temperature is raised. The dye is readily soluble in methyl alcohol, less easily in absolute alcohol, changing to orange. It is somewhat soluble in boiling acetic acid, difficultly in cold water, the color changing to bright orange-red owing to formation of the hydrate. It dissolves readily in boiling water and gives a bright red color with conc. sulfuric acid.

Subs., air-dry, 0.3140: loss, 0.0338 *in vacuo* at  $100^{\circ}$  over  $\text{H}_2\text{SO}_4$ .

Calc. for  $\text{C}_{14}\text{H}_{14}\text{O}_5\text{N}_2\text{S} \cdot 2\text{H}_2\text{O}$ :  $\text{H}_2\text{O}$ , 10.06. Found: 10.76.

Subs., anhydrous, 0.1364: 10.55 cc. N ( $30.5^{\circ}$ , 755 mm.).

Calc. for  $\text{C}_{14}\text{H}_{14}\text{O}_5\text{N}_2\text{S}$ : N, 8.70. Found: 8.64.

*4-Amino-6-ethoxy-phenol*,  $4,6\text{-H}_2\text{N}(\text{C}_2\text{H}_5\text{O})\text{C}_6\text{H}_3\text{OH}$ .—Seventy-two g. of the crude dye were dissolved in 720 cc. of 10% aqueous ammonia and treated with a rapid stream of hydrogen sulfide. The solution became hot, suddenly decolorized, and then began to deposit the aminophenol. After passing in hydrogen sulfide for  $\frac{1}{2}$  hour the mixture was cooled with ice-water and the aminophenol filtered off and dried. The yield was 29 g. Recrystallization from 85% alcohol yielded an impure product, apparently owing to partial oxidation, so a portion of the substance was suspended in a little water, dissolved by adding hydrochloric acid, and the solution treated with sodium acetate. The resulting precipitate of dark, flocculent material was filtered off with the aid of bone black and the filtrate neutralized with sodium hydrogen carbonate. Under these conditions the compound separated as almost colorless, minute, glistening, hexagonal platelets melting to a deep brown liquid at  $186\text{--}8^{\circ}$  with preliminary darkening and softening. The aminophenol is readily soluble in

hot acetone, less easily in the cold, and dissolves in boiling alcohol and only sparingly in the cold. It is rather sparingly soluble in boiling water and dissolves in alkali with a gray lilac color, changing to deep violet. An alcoholic solution gives an olive color with ferric chloride. The substance also turns purple with sulfuric acid, but dissolves with very little color.

Subs., 0.1493: (Kjeldahl), 9.7 cc. 0.1 *N* HCl.

Calc. for  $C_8H_{11}O_2N$ : N, 9.15. Found: 9.10.

*4-Acetamino-6-ethoxy-phenol*.—Twenty-nine g. of the crude aminophenol were dissolved in 145 cc. of warm 50% acetic acid, chilled in ice-water, and shaken with 23 g. (1.2 mols.) of acetic anhydride. The acetyl derivative crystallized on rubbing, and after diluting with a little water and letting stand in the ice box the collected product was recrystallized from 50% alcohol. The yield was 23.4 g. Recrystallized first from water containing a few drops of acetic acid, using bone black, then from 50% acetic acid, the acetaminophenol forms practically colorless, nacreous platelets which melt at 165.5–6.5° with preliminary softening. It is readily soluble in alcohol at room temperature, the solution giving an olive color with ferric chloride. It is very difficultly soluble in cold water, easily on boiling, an aqueous suspension giving an orange color with ferric chloride. The substance is soluble in warm acetone, sparingly in the cold, and is also slightly soluble in boiling benzene.

Subs., 0.1875: (Kjeldahl), 9.8 cc. 0.1 *N* HCl.

Calc. for  $C_{10}H_{13}O_3N$ : N, 7.19. Found: 7.32.

*4-Chloroacetyl-amino-6-ethoxy-phenol*.—In this case it was necessary to add 6 additional parts of 50% acetic acid to the usual acetic acid-sodium acetate mixture before a clear solution could be obtained. The crude chloroacetyl derivative was recrystallized from 50% alcohol, in which it is easily soluble at the boiling point and only sparingly so a few degrees below. The yield was equal to the amount of aminophenol taken. Recrystallized again from boiling toluene it forms woolly needles which melt at 155–6° with preliminary softening. It dissolves readily in acetone and in boiling chloroform or alcohol, less easily in the last two in the cold. It also dissolves in



cold methyl alcohol or boiling water, and is rather sparingly soluble in boiling toluene.

Subs., 0.1546: (Kjeldahl), 6.55 cc. 0.1 *N* HCl.

Calc. for  $C_{10}H_{12}O_3NCl$ : N, 6.11. Found: 5.93.

*4-Acetamino-guaiacol*.—Forty g. of 4-amino-guaiacol<sup>1</sup> were dissolved in 200 cc. of 50% acetic acid and shaken with 1.2 molecular equivalents of acetic anhydride. The resulting solution was concentrated to dryness *in vacuo* and taken up in the minimum amount of water. The acetyl derivative crystallized on rubbing and letting stand, and was filtered off, dried, and recrystallized by dissolving in hot ethyl acetate, treating with bone black, and precipitating with the aid of ether. The yield was 25 g., the substance corresponding in its properties with that reported in the literature.<sup>2</sup> An additional amount was recovered by concentrating the mother liquors and adding ether.

*4-Amino-veratrol*.—12.5 g. of 4-acetamino-guaiacol were methylated in the usual way with dimethyl sulfate and potassium hydroxide solution, saturating with salt at the end to complete the separation of the acetamino-veratrol. This was filtered off, washed with a little ice-water, and saponified by boiling for 50 minutes with 25% sulfuric acid. The solution was chilled, made strongly alkaline, and the crystalline base filtered off and washed first with saturated sodium chloride solution, then with a little ice-water. The yield was 7.5 g., corresponding in its properties with those recorded in the literature.

*3,4-Dimethoxy-chloroacetanilide*.—Five g. of 4-amino-veratrol were chloroacetylated as in previous experiments. After recrystallization from 50% alcohol the yield was 6.2 g. Recrystallized from benzene it forms long, silky needles which melt constantly at 133.5–4.5° with slight preliminary softening. The compound dissolves readily in chloroform or acetone, rather sparingly in cold alcohol, easily on warming, and also dissolves in boiling water or boiling benzene.

Subs., 0.1655: (Kjeldahl), 7.35 cc. 0.1 *N* HCl.

Calc. for  $C_{10}H_{12}O_3NCl$ : N, 6.11. Found: 6.22.

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<sup>1</sup> *Loc. cit.*

<sup>2</sup> *Ber.*, 39, 3340 (1906); *Chem. Centr.*, 1911, II, 1437.

*3-Methoxy-4-ethoxy-acetanilide*,  $3,4\text{-CH}_3\text{O}(\text{C}_2\text{H}_5\text{O})\text{C}_6\text{H}_3\text{NHCOCH}_3$ .—16.7 g. of 4-acetamino-guaiacol were dissolved in the minimum amount of boiling water and treated with 95 cc. of *N* potassium hydroxide solution, the temperature dropping to  $45^\circ$ . Twelve g. of diethyl sulfate were then added in small portions, with vigorous shaking, the temperature remaining at  $45\text{--}50^\circ$  and the ether separating before all of the diethyl sulfate had been added. After shaking for a few minutes longer 50 cc. more of *N* potassium hydroxide solution were added, the temperature was raised to  $50^\circ$ , and the mixture then shaken with an additional 6 cc. of diethyl sulfate. After treating with aqueous ammonia to decompose any unchanged diethyl sulfate, the mixture was cooled, allowed to stand, and the product filtered off and dried. The yield was 15.8 g. A portion was recrystallized first from water, in which the colored impurities remained soluble, and then from toluene, separating as long, narrow, nacreous plates which melt at  $148.5\text{--}50^\circ$  with slight preliminary softening. The compound separates from water as thick plates and columns. It also dissolves in alcohol or acetone at room temperature, and dissolves readily in hot benzene and freely, but slowly, in boiling toluene.

Subs., 0.1518: (Kjeldahl), 7.4 cc. 0.1 *N* HCl.

Calc. for  $\text{C}_{11}\text{H}_{13}\text{O}_3\text{N}$ : N, 6.70. Found: 6.83.

As stated in the introduction, this substance appears to have been obtained by Freyss.<sup>1</sup>

*3-Methoxy-4-ethoxy-aniline*.—14.5 g. of the acetamino compound were boiled for 55 minutes with 70 cc. of 25% sulfuric acid. The mixture was cooled, causing the sulfate to separate as large plates, and was made strongly alkaline and shaken out with ether. After drying the ethereal extract, concentrating, and fractionating the residue *in vacuo*, 6 g. of the base were obtained. It is a faintly yellow, very viscous liquid, which boils at  $175\text{--}6^\circ$  under a pressure of 20 mm. and soon solidifies to a mass of prismatic needles which show a solidification point of  $55^\circ$  (corr.) when the thermometer is placed in the crystallizing liquid. A few of the crystals, crushed on a porous plate, melted with preliminary softening at  $55^\circ$  to a turbid liquid

<sup>1</sup> *Loc. cit.*

which cleared completely at 59°. The crystalline base dissolves readily at room temperature in alcohol, benzene, or ether, and only sparingly in ligroin. It is difficultly soluble in water, the solution turning brown with ferric chloride and changing through wine-red to reddish purple on standing. From a solution in an excess of dil. hydrochloric acid the hydrochloride separates on chilling as very thin, nacreous scales. It is readily diazotizable, the purple-red diazo solution (brownish green in thin layers) coupling with R-salt to give an intense purple-red color.

Subs., 0.1337: (Kjeldahl), 7.8 cc. 0.1 *N* HCl.

Calc. for  $C_9H_{13}O_2N$ : N, 8.39. Found: 8.19.

*3-Methoxy-4-ethoxy-chloroacetanilide*.—3.5 g. of the base were chloroacetylated in a mixture of 45 cc. of 50% acetic acid and 15 cc. of saturated sodium acetate solution. After diluting with water and washing the collected product the yield was 4 g. Recrystallized from a small volume of 50% alcohol, in which it is easily soluble at the boiling point and very much less so on cooling even a few degrees, the substance forms long, silky needles which melt slowly at 133–4° with slight preliminary softening. It dissolves readily in acetone or chloroform, less easily in the cold in alcohol or benzene, and is fairly readily soluble in boiling water.

Subs., 0.1586: (Kjeldahl), 6.7 cc. 0.1 *N* HCl.

Calc. for  $C_{11}H_{14}O_3NCl$ : N, 5.75. Found: 5.92.

*4-Methoxy-5-ethoxy-acetanilide*, 4,5- $CH_3O(C_2H_5O)C_6H_2NHCOCH_3$ .—Eighteen g. of 4-acetamino-6-ethoxy-phenol (see p. 299) were dissolved in 100 cc. of *N* potassium hydroxide solution and the deep blue solution methylated in the usual way by means of dimethyl sulfate. The methyl ether separated almost at once and the yield of crude product was 18.3 g. A portion was recrystallized first from water, containing a few drops of acetic acid, using bone black, then from toluene, forming slightly purple, very thin, nacreous scales which melt slowly and constantly at 145–6° with preliminary softening. A mixture with the 3,4-isomer, which melts only a few degrees higher, softens and gradually melts above 115°, becoming entirely clear at 138°. The compound dissolves freely at room temperature in

alcohol, acetone, or chloroform, and is sparingly soluble in cold toluene, easily on boiling. It is also difficultly soluble in cold water, but fairly readily so at the boiling point.

Subs., 0.1495: (Kjeldahl), 7.15 cc. 0.1 *N* HCl.

Calc. for  $C_{11}H_{15}O_3N$ : N, 6.70. Found: 6.70.

*4-Methoxy-5-ethoxy-aniline*.—17.5 g. of the acetamino compound were boiled with 10 parts of 25% sulfuric acid. The clear, dark purple solution was cooled in a freezing mixture, made strongly alkaline, and extracted with ether. After drying and concentrating, the crystalline residue was taken up in boiling benzene and the solution treated with ligroin until the initial turbidity barely disappeared. 10.2 g. of the base crystallized on seeding. Recrystallized from water with the aid of bone black, then from 50% alcohol, the substance separates slowly as faintly pinkish, rhombic crystals which melt constantly at  $81.5-2^\circ$  (corr.), with slight preliminary softening. It dissolves very easily in acetone or benzene, somewhat less readily in alcohol or ether, and only sparingly in 50% alcohol at  $0^\circ$ , but readily on warming. It is quite soluble in boiling water and rather difficultly in the cold, the aqueous solution gradually giving an intense violet color with ferric chloride. A solution of the base in 1:1 hydrochloric acid soon deposits the hydrochloride as delicate, colorless needles. In a dil. hydrochloric acid solution of the amine sodium nitrite gives a transient purple color, brown in thin layers, changing to brownish gray, and the substance couples with R-salt to give a deep red color.

Subs., 0.1688: (Kjeldahl), 10.3 cc. 0.1 *N* HCl.

Calc. for  $C_9H_{13}O_2N$ : N, 8.39. Found: 8.55.

*4-Ethoxy-5-ethoxy-chloroacetanilide*.—5.1 g. of the base were dissolved in a mixture of 50 cc. of 50% acetic acid and 25 cc. of saturated sodium acetate solution, diluted with 100 cc. of 25% acetic acid, and treated with 4.3 cc. of chloroacetyl chloride, with chilling and stirring. The chloroacetyl derivative, which separated immediately, was filtered off and recrystallized from alcohol, the yield being 5.6 g. Recrystallized again from toluene it forms delicate, woolly needles which melt constantly at  $135.5-6^\circ$  with slight preliminary softening. It



dissolves readily in acetone or chloroform, less easily in alcohol, and difficultly in cold toluene or benzene, but readily on boiling. It is rather sparingly soluble in boiling water. In its properties, therefore, it closely resembles the 3-methoxy-4-ethoxy-isomer.

Subs., 0.1578: (Kjeldahl), 6.4 cc. 0.1 *N* HCl.

Calc. for  $C_{11}H_{14}O_3NCl$ : N, 5.75. Found: 5.68.

*Diacetyl-4-amino-pyrocatechol*.—Twenty-five g. of crude 4-amino-pyrocatechol hydrobromide<sup>1</sup> were purified by solution in water containing a few drops of hydrobromic acid, treating with a few grams of stannous chloride, detinning with hydrogen sulfide, and concentrating the solution to dryness *in vacuo*. The residue was taken up in 75 cc. of water, treated with 90 cc. of saturated sodium acetate solution, and shaken with 14 cc. (1.1 mols.) of acetic anhydride. The resulting mixture was cooled and filtered and the filtrate treated with solid sodium acetate and again shaken with 5 cc. of acetic anhydride. An additional amount of the diacetyl derivative separated and was filtered off, washed with a little water, and combined with the first fraction, the total yield being 14.4 g. Recrystallized twice from 50% alcohol containing a few drops of acetic acid, the substance separates as thin, glistening, faintly pinkish, hexagonal platelets which melt constantly at 187.5–92°. The compound dissolves readily in acetone, rather sparingly in 50% or 95% alcohol at room temperature, and difficultly in cold water, readily on boiling. An aqueous solution gives a grayish brown color with ferric chloride. An aqueous suspension dissolves on adding a drop of dil. sodium carbonate or ammonia, the solution in the latter case turning rose-brown on shaking. When an aqueous suspension is warmed with sodium nitrite and a few drops of acetic acid the resulting clear solution deposits, on cooling and scratching, golden yellow platelets of a nitroso derivative which dissolves in alkalies with a brown-red color, changing rapidly to purple-red.

Subs., 0.1598: (Kjeldahl), 7.85 cc. 0.1 *N* HCl.

Calc. for  $C_{10}H_{11}O_4N$ : N, 6.70. Found: 6.88.

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<sup>1</sup> THIS JOURNAL, 41, 467 (1919).

*3,4-Diethoxy-acetanilide*,  $3,4-(C_2H_5O)_2C_6H_3NHCOCH_3$ .—Thirteen g. of diacetyl-4-amino-pyrocatechol were suspended in about 150 cc. of water in a flask provided with a 3-hole stopper through which passed a gas delivery tube, an exit tube, and a dropping funnel. After the air in the flask had been displaced by hydrogen, 110 cc. of 2 *N* potassium hydroxide solution (a little over 3 mols.) were added through the dropping funnel, followed, after complete solution had taken place, by 16.5 cc. (2 mols.) of diethyl sulfate. The mixture was then warmed on the water bath, shaking continuously and passing in a stream of hydrogen. The diethoxy-acetanilide finally separated in crystalline form, after which 20 cc. of 5 *N* potassium hydroxide solution and 9 cc. of diethyl sulfate were added and the mixture again shaken and heated. After all of the diethyl sulfate had apparently been used up ammonia was added and the mixture allowed to cool slowly. It was finally chilled in ice, filtered, and the crude product recrystallized from a small volume of 50% alcohol. The yield was 4.9 g., agreeing in all of its properties with the substance as obtained by the alternative method described below and causing no sensible depression of the melting point when mixed with this.

4-Acetamino-6-ethoxy-phenol (see p. 299) was ethylated in exactly the same manner as described for the ethylation of 4-acetamino-guaia-col (see p. 300). The yield of crude 3,4-diethoxy-acetanilide obtained in this way was slightly more than the amount of starting material used, and this method is therefore recommended when guethol is available, as it is more direct and the yield is better. Recrystallized from 50% alcohol it forms nacreous platelets which melt at 124–5.5° with slight preliminary softening, thus agreeing with the description given by Wisinger,<sup>1</sup> who obtained the compound by reduction of nitro-pyrocatechol diethyl ether, acetylation of the amine hydrochloride, and saponification of the diacetyl derivative so obtained. Wisinger was unable to isolate the free base in a state of purity, but as will be seen below, this offers little difficulty.

Subs., 0.1740: (Kjeldahl), 7.75 cc. 0.1 *N* HCl.

Calc. for  $C_{12}H_{17}O_3N$ : N, 6.28. Found: 6.24.

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<sup>1</sup> *Monatsh.*, **21**, 1015 (1900).

*3,4-Diethoxy-aniline*.—6.8 g. of the diethoxy-acetanilide were boiled for 15 minutes with 35 cc. of 1:1 hydrochloric acid, diluted with 2 parts of water, cooled somewhat, made strongly alkaline, chilled rapidly, and the mixture extracted with ether. The ethereal solution, when dried and concentrated, yielded 4.8 g. of the base as a crystalline residue. Recrystallized first by dissolving in benzene, adding ligroin, and letting stand in the ice box, then from a relatively large volume of ligroin, it separates as cream-colored prisms, rhombs, thick plates, and needles which melt constantly at 47.5–8.5° (corr.). It is readily soluble in the cold in the usual neutral organic solvents with the exception of ligroin, and is also appreciably soluble in cold water. The aqueous solution gives an intense violet color with ferric chloride. When the base is dissolved in warm, dil. hydrochloric acid, the hydrochloride separates on cooling as delicate needles. A solution of the salt gives a purple color with sodium nitrite, coupling with R-salt to give a purple-red color.

Subs., 0.1513: (Kjeldahl), 8.4 cc. 0.1 *N* HCl.

Calc. for  $C_{10}H_{13}O_2N$ : N, 7.74. Found: 7.78.

*3,4-Diethoxy-chloroacetanilide*.—The base was dissolved in a mixture of 15 parts of 50% acetic acid and 3 parts of saturated sodium acetate solution and chloroacetylated in the usual way. Precipitation of the chloroacetyl derivative was completed by dilution with water. Recrystallized first from 85% alcohol, then from toluene, it forms hair-like needles which melt at 122.5–4.5° with slight preliminary softening and resolidification a few degrees below the melting point. The substance dissolves readily in acetone or chloroform, less easily in cold alcohol, and only sparingly in cold toluene although very readily on boiling. It is practically insoluble in cold water, appreciably so on boiling, and dissolves in conc. sulfuric acid with a faint greenish yellow color.

Subs., 0.1529: (Kjeldahl), 6.15 cc. 0.1 *N* HCl.

Calc. for  $C_{12}H_{11}O_3NCl$ : N, 5.44. Found: 5.63.

(C) *Derivatives of the Ethers of Resorcinol.*

*p*-Sulfo-phenylazo-*m*-methoxy-phenol,  $p\text{-HO}_3\text{SC}_6\text{H}_4\text{N:NC}_6\text{H}_3(\text{OCH}_3)\text{-OH}(o',p')\text{-}$ .—This substance was prepared exactly in the same way as was the *p*-sulfo-phenylazo-*o'*-ethoxy-phenol described above (p. 297), using 12.4 g. of resorcinol monomethyl ether. 25.2 g. of the dye separated as an orange-red crystalline precipitate on strongly acidifying the reaction mixture with hydrochloric acid. A portion of the dye was recrystallized from water, in which it is sparingly soluble in the cold, quite easily on boiling, separating as lustrous, brown-orange, lenticular platelets. After washing with water, then with acetone, and air-drying, the compound retained approximately one molecule of water of crystallization. The anhydrous substance forms a brick-red powder which gradually darkens on heating and chars and swells at about 250°. It is fairly readily soluble in absolute alcohol, finally crystallizing out again on warming as short, thick, orange, microscopic plates. It is rather sparingly soluble in boiling acetic acid, a portion separating from the hot solvent in this case as well, apparently owing to combination with the solvent. The dye dissolves in conc. sulfuric acid with a yellow-orange color, in dil. carbonates or alkalis with a reddish orange color.

Subs., air-dry, 0.2534: loss, 0.0163 *in vacuo* at 100° over  $\text{H}_2\text{SO}_4$ .

Calc. for  $\text{C}_{13}\text{H}_{12}\text{O}_5\text{N}_2\text{S}\cdot\text{H}_2\text{O}$ :  $\text{H}_2\text{O}$ , 5.52. Found: 6.43.

Subs., anhydrous, 0.1293: 9.8 cc. N (24.5°, 770 mm.).

Calc. for  $\text{C}_{13}\text{H}_{12}\text{O}_5\text{N}_2\text{S}$ : N, 9.09. Found: 8.82.

*4*-Amino-5-methoxy-phenol,  $4,5\text{-H}_2\text{N}(\text{CH}_3\text{O})\text{C}_6\text{H}_3\text{OH}$ .—Twenty-four g. of *p*-sulfo-phenylazo-*m*-methoxy-phenol were dissolved in 240 cc. of 10% ammonium hydroxide and treated with a rapid stream of hydrogen sulfide until the solution became brown and the amino-phenol was precipitated. After cooling, the precipitate, white at first, was filtered off and washed with water, rapidly turning gray on exposure to air. After drying *in vacuo* the yield was 7.6 g. Recrystallized from boiling toluene, in which it is sparingly soluble, the base separates as delicate, pale purple-brown needles which darken when heated, blacken markedly at about 160°, and then soften, finally melting at 175–80° to a purple-black liquid. The substance



dissolves in alcohol, but is very sparingly soluble in cold water, although readily on boiling, the solution turning purple in the air. An aqueous suspension gives a slowly developing brownish purple color with ferric chloride.

Subs., 0.1500: (Kjeldahl), 10.8 cc. 0.1 *N* HCl.

Calc. for  $C_7H_7O_2N$ : N, 10.07. Found: 10.08.

The hydrochloride of this substance was obtained by Heinrich and Rhodius<sup>1</sup> by reduction of nitroso-resorcinol monomethyl ether with stannous chloride, but the free base was not isolated. The isomeric resorcinol derivative containing the amino group in the position *para* to the methoxy group, was obtained by Bechhold<sup>2</sup> by reduction of the phenolazo-resorcin monomethyl ether formed on methylation of phenylazo-resorcinol.

*4-Acetamino-5-methoxy-phenol*.—4-Amino-5-methoxy-phenol was acetylated in 50% acetic acid solution by means of acetic anhydride. The resulting solution was concentrated to dryness *in vacuo*, the residue taken up in hot water, and the solution treated with bone black and filtered. The acetyl derivative separated on standing overnight. Recrystallized with the aid of bone black from water containing a few drops of acetic acid, then from toluene, which removes a black impurity, the acetaminophenol separates as pale pink aggregates of minute needles which begin to melt above 140° when slowly heated and finally melt completely at 169–71.5°. When rapidly heated the substance melts at 150–5°, resolidifies in a few moments, and then melts again at 169–71°. It is soluble in cold alcohol or acetone and dissolves rather sparingly in cold water, readily on boiling, separating on cooling as radiating masses or spears.

Subs., 0.1571: (Kjeldahl), 8.70 cc. 0.1 *N* HCl.

Calc. for  $C_9H_{11}O_3N$ : N, 7.74. Found: 7.76.

*4-Chloroacetyl-amino-5-methoxy-phenol*.—Five g. of crude 4-amino-5-methoxy-phenol were dissolved in 60 cc. of 50% acetic acid and treated with bone black. The filtrate was treated with 25 cc. of saturated sodium acetate solution and chloroacetylated in the usual

<sup>1</sup> *Ber.*, **35**, 1485 (1902).

<sup>2</sup> *Ibid.*, **22**, 2378 (1889).

way. After recrystallization from 50% alcohol the yield of the acyl derivative was 5.1 g. Recrystallized again from ethyl acetate it forms practically colorless, nacreous platelets which melt constantly at  $165.5-6.5^{\circ}$  with preliminary softening. The substance is easily soluble in cold alcohol or acetone, also in boiling ethyl acetate or water, but sparingly in the cold. It is also very difficultly soluble in boiling chloroform.

Subs., 0.1534: (Kjeldahl), 7.25 cc. 0.1 *N* HCl.

Calc. for  $C_9H_{10}O_3NCl$ : N, 6.50. Found: 6.53.

*p*-Sulfo-phenylazo-*m*'-ethoxy-phenol.—This substance was prepared exactly as given for the analogous dyes already described, using 13.8 g. resorcinol monoethyl ether. The product separated slowly on acidifying strongly with hydrochloric acid, and was filtered off after standing in the refrigerator overnight, washed with ice-water and acetone, and dried. The yield was 25.4 g. Recrystallized from water, it forms flat, minute, glistening, brown-orange, pointed needles and narrow plates with bevelled edges. After washing with acetone and air-drying, the substance contained approximately one molecule of water of crystallization. The anhydrous substance forms a brick-red powder which blackens at about  $250-5^{\circ}$  and softens on further heating, but does not melt below  $285^{\circ}$ . It is difficultly soluble in boiling absolute alcohol or acetic acid and is practically insoluble in boiling acetone. In sulfuric acid it gives a bright orange color. It dissolves rather sparingly in cold water with the same color, becoming slightly more red on adding alkali.

Subs., air-dry, 0.5054: loss, 0.0306 *in vacuo* at  $100^{\circ}$  over  $H_2SO_4$ .

Calc. for  $C_{14}H_{14}O_5N_2S \cdot H_2O$ :  $H_2O$ , 5.30. Found: 6.06.

Subs., anhydrous, 0.1197: 9.05 cc. N ( $23.5^{\circ}$ , 759 mm.).

Calc. for  $C_{14}H_{14}O_5N_2S$ : N, 8.70. Found: 8.70.

4-Amino-5-ethoxy-phenol.—*p*-Sulfo-phenylazo-*m*-ethoxy-phenol was reduced by hydrogen sulfide in ammoniacal solution as in previous experiments. The yield of aminophenol was about 40% of the dye used. A portion was rapidly recrystallized from water containing hydrogen sulfide to suppress the oxidation which otherwise occurred, forming gray, glistening, microscopic leaflets which melt at  $152-4^{\circ}$

with preliminary softening and blackening. The compound dissolves readily in alcohol, less easily in cold acetone, readily on boiling, and is sparingly soluble in hot chloroform. It dissolves in boiling toluene with the exception of a few dark flocks, but on attempting to purify a portion of the substance by this method it darkened rapidly owing to oxidation. An aqueous suspension gives a purple color with ferric chloride, deepening to an intense violet, while a solution of the compound in dil. alkali rapidly turns dark purple and gives a dark purple precipitate.

Subs., 0.1530: (Kjeldahl), 10.0 cc. 0.1 *N* HCl.

Calc. for  $C_8H_{11}O_2N$ : N, 9.15. Found: 9.16.

*4-Acetamino-5-ethoxy-phenol*.—Twenty g. of 4-amino-5-ethoxy-phenol were dissolved in 240 cc. of 50% acetic acid, treated with bone black, and the filtrate shaken with 15.2 cc. of acetic anhydride. After several hours in the cold the acetaminophenol was filtered off and washed with water. The yield was 20.6 g., an additional 2.1 g. being obtained on concentration of the mother liquors. A portion was recrystallized first from 60% alcohol, then from 50% acetic acid, separating on seeding as pointed prisms of various shapes which melt slowly at 172.5–4.5° with preliminary softening, the melting point being unchanged by a subsequent recrystallization. The substance is fairly readily soluble in cold alcohol, acetone, or ethyl acetate, and is very difficultly soluble in cold water, although quite soluble on boiling.

Subs., 0.1839: (Kjeldahl), 9.3 cc. 0.1 *N* HCl.

Calc. for  $C_{10}H_{13}O_3N$ : N, 7.19. Found: 7.09.

*4-Chloroacetyl-amino-5-ethoxy-phenol*.—4-Amino-5-ethoxy-phenol was chloroacetylated exactly as in the case of the 4, 6-isomer (p. 299). Recrystallized first from 50% alcohol containing a few drops of acetic acid, using bone black, the substance separates as gray, felted needles, while after a subsequent recrystallization from toluene it forms nacreous, feathery aggregates of gray plates which melt slowly at 158.5–61°. It dissolves readily in alcohol or acetone, less easily in chloroform, and is rather difficultly soluble in boiling water and practically insoluble in the cold. An alcoholic solution gives an olive color with ferric chloride.

Subs., 0.1523: (Kjeldahl), 9.45 cc. 0.714  $N^1$  HCl.

Calc. for  $C_{10}H_{12}O_3NCl$ : N, 6.11. Found: 6.20.

*2,4-Dimethoxy-aniline*, 2,4- $(CH_3O)_2C_6H_3NH_2$ .—This substance was prepared by methylating 4-acetamino-5-methoxy-phenol with aqueous alkali and dimethyl sulfate and hydrolyzing the resulting 2,4-dimethoxy-acetanilide by boiling for  $\frac{1}{2}$  hour with 1:1 hydrochloric acid, just as was done in the preparation of 4-amino-veratrol from 4-acetamino-guaiacol. The solution was made strongly alkaline and the base extracted with ether. After drying, this was concentrated to small bulk, the residue crystallizing in a freezing mixture. The product was melted, taken up in several volumes of ligroin, and the solution chilled and seeded. The base separated as nacreous, pinkish platelets which melted at 32.5–3.5° (corr.), the melting point being unchanged by subsequent recrystallization from a mixture of benzene and ligroin with the aid of a freezing mixture. According to Bechhold,<sup>2</sup> who prepared the base by reduction of phenylazo-resorcinol dimethyl ether, it melts at 39–40°. An aqueous solution gives a deep purple color with ferric chloride, while an alcoholic solution gives a green color, gradually changing to violet-brown.

Subs., 0.1226: (Kjeldahl), 7.9 cc. 0.1  $N$  HCl.

Calc. for  $C_8H_{11}O_2N$ : N, 9.15. Found: 9.03.

The hydrochloride of this substance was also prepared by Kauffmann and Kugel<sup>3</sup> by reduction of the nitro compound with tin and hydrochloric acid.

*2,4-Dimethoxy-chloroacetanilide*.—This compound was prepared from the base as in previous examples and recrystallized from 50% alcohol, the yield being almost quantitative. It forms delicate needles which melt at 89.5–90° (corr.) with slight preliminary softening. It dissolves readily in the usual organic solvents in the cold, with the exception of ligroin, in which, however, it is appreciably soluble in the cold and readily on heating. It is almost insoluble in cold water but dissolves fairly readily on boiling.

<sup>1</sup> Equal to  $\frac{1}{14}$  normal.

<sup>2</sup> *Loc. cit.*

<sup>3</sup> *Ber.*, **44**, 2387 (1911).



Subs., 0.1310: (Kjeldahl), 5.7 cc. 0.1 *N* HCl.

Calc. for  $C_{10}H_{12}O_3NCl$ : N, 6.11. Found: 6.09.

*2-Methoxy-4-ethoxy-acetanilide*,  $2,4-CH_3O(C_2H_5O)C_6H_3NHCOCH_3$ .—4-Acetamino-5-methoxy-phenol was ethylated by means of diethyl sulfate and aqueous potassium hydroxide in warm solution as described in previous examples. Recrystallized first with the aid of bone black from 25% alcohol containing a few drops of acetic acid, then by dissolving in hot benzene and treating the solution with an equal volume of ligroin, the substance forms pale pink, glistening platelets which melt slowly and constantly at  $117.5-8.5^\circ$  with slight preliminary softening. It dissolves readily in the cold in alcohol, acetone, or chloroform, less easily in ether or benzene, and difficultly in ligroin or water. It is quite soluble in boiling water and dissolves in conc. sulfuric acid with a faint pink color.

Subs., 0.1536: (Kjeldahl), 10.25 cc. 0.0714 *N* HCl.

Calc. for  $C_{11}H_{15}O_3N$ : N, 6.70. Found: 6.67.

*2-Methoxy-4-ethoxy-aniline*.—The acetamino compound was hydrolyzed by boiling for  $\frac{1}{2}$  hour with 1:1 hydrochloric acid and the base isolated by shaking out the alkaline solution with ether and fractionating the residue *in vacuo*. The yield was good. The amine forms a very viscous, practically colorless oil, which boils at  $151.5-2.5^\circ$  under a pressure of 12 mm. and solidifies on chilling and rubbing. A portion was dissolved in a little benzene, diluted with ligroin, chilled, and let stand in the ice box after seeding, separating as faintly pinkish rhombs which melt at  $27.5-8.5^\circ$  (corr.). It dissolves readily in the usual organic solvents, less easily in ligroin. It is quite soluble in water, the aqueous solution giving a violet-purple color with ferric chloride, finally depositing purple, microscopic needles. The base dissolves in dil. hydrochloric acid, separating from a sufficiently concentrated solution on rubbing as needles and short prisms. It is readily diazotized, coupling with R-salt to give a deep, purple-red color.

Subs., 0.1860: (Kjeldahl), 15.35 cc. 0.0714 *N* HCl.

Calc. for  $C_9H_{13}O_2N$ : N, 8.39. Found: 8.25.

*2-Methoxy-4-ethoxy-chloroacetanilide*.—This compound was prepared as in previous examples. Recrystallized twice from a small

volume of 85% alcohol, then from ligroin, it forms flat, narrow, striated plates which soften slightly at 97° and melt slowly at 97.5–8.0°. It is very easily soluble at room temperature in acetone, chloroform, benzene, or toluene, less readily in alcohol, and is almost insoluble in cold water, more easily on boiling.

Subs., 0.1615: (Kjeldahl), 9.15 cc. 0.0714 *N* HCl.

Calc. for  $C_{11}H_{14}O_3NCl$ : N, 5.75. Found: 5.66.

*4-Methoxy-6-ethoxy-acetanilide*, 4,6- $CH_3O(C_2H_5O)C_6H_3NHCOCH_3$ .—4-Acetamino-5-ethoxy-phenol (p. 310) was methylated in the usual way with dimethyl sulfate and aqueous potassium hydroxide. When recrystallized first from 25% alcohol containing a few drops of acetic acid, with the aid of bone black, then from ligroin, the substance separates as faintly pink, silky needles which melt at 100.5–1.0° with slight preliminary softening. It dissolves readily in alcohol, benzene, or acetone, less easily in ether, and is very difficultly soluble in cold ligroin, more easily on boiling. It dissolves sparingly in cold water, quite readily on boiling. The substance gives a faint yellow color with conc. sulfuric acid.

Subs., 0.2209: (Kjeldahl), 14.9 cc. 0.0714 *N* HCl.

Calc. for  $C_{11}H_{13}O_3N$ : N, 6.70. Found: 6.75.

*4-Methoxy-6-ethoxy-aniline*.—11.3 g. of the acetamino compound were hydrolyzed with 1:1 hydrochloric acid. The chilled solution was decanted from the crystals of the amine hydrochloride, which were taken up in hot water and the solution cooled, made strongly alkaline, and shaken out with ether. After drying over potassium hydroxide and concentrating, the residue was fractionated *in vacuo*. 7.5 g. were obtained as a pale straw-colored, viscous liquid which boils at 144–4.5° under a pressure of 9 mm. and solidifies when chilled to a mass of thin platelets which melt at 22.5°. It is readily soluble in the usual organic solvents and dissolves appreciably in water at room temperature, readily on heating. An aqueous solution gives a brownish color with ferric chloride, changing to dark purple and giving a precipitate of the same color. In dil. hydrochloric acid it gives a bluish solution with sodium nitrite, but the solution contains the diazo salt, as it couples with R-salt to give a red color.

Subs., 0.1455: (Kjeldahl), 11.75 cc. 0.0714 *N* HCl.

Calc. for  $C_9H_{13}O_2N$ : N, 8.39. Found: 8.08.

*4-Methoxy-6-ethoxy-chloroacetanilide*.—The base was chloroacetylated in the usual way. Recrystallized from 50% alcohol, then from toluene, it forms thick, almost colorless, glistening platelets which melt at  $126-7^\circ$  with slight preliminary softening. It is very easily soluble in chloroform, quite soluble in acetone, and rather sparingly in cold alcohol, but readily on boiling.

Subs., 0.1517: (Kjeldahl), 8.45 cc. 0.0714 *N* HCl.

Calc. for  $C_{11}H_{14}O_3NCl$ : N, 5.75. Found: 5.57.

*2,4-Diethoxy-acetanilide*,  $2,4-(C_2H_5O)_2C_6H_3NHCOCH_3$ .—6.8 g. of 4-acetamino-5-ethoxy-phenol were ethylated as in previous experiments. The yield was 4.5 g., melting at  $117-8^\circ$ . Recrystallized from 50% alcohol, the diethyl ether forms silky needles of the same melting point. Will and Pukall,<sup>1</sup> who prepared the substance by reduction of phenylazo-resorcinol diethyl ether and acetylation of the amine hydrochloride, give  $120.5^\circ$  as the melting point. The compound is very difficultly soluble in cold water but dissolves quite freely on boiling, the undissolved portion melting to an oil.

*2,4-Diethoxy-aniline*.—The crude acetamino compound was hydrolyzed and the free base isolated as in previous examples. The residue from the ether extraction solidified on letting stand in a freezing mixture with occasional rubbing. It was again melted, taken up in a very little benzene, and diluted with several volumes of ligroin, chilled in a freezing mixture, and seeded. The base separated as pale brownish pink, flat needles and narrow platelets which melt at  $33.5-4.0^\circ$  (corr.) with preliminary softening. Will and Pukall give  $32^\circ$  as the melting point. The base seems quite stable in the air, and is smoothly diazotizable, coupling with R-salt to give a purplish red color. An aqueous solution gives a slowly developing, deep violet color with ferric chloride, followed by a precipitate of dark violet microscopic needles.

Subs., 0.1330: (Kjeldahl), 7.3 cc. 0.1 *N* HCl.

Calc. for  $C_{10}H_{10}O_2N$ : N, 7.74. Found: 7.69.

<sup>1</sup> *Ber.*, 20, 1127 (1887).

*2,4-Diethoxy-chloroacetanilide*.—One g. of the base was dissolved in a mixture of 5 cc. of acetic acid and 5 cc. of saturated sodium acetate solution; diluted with 30 cc. of 50% acetic acid, and chloroacetylated in the usual way. After dilution the product was filtered off and recrystallized from 85% alcohol, separating as delicate, woolly needles, which melt at 102–3° with slight preliminary softening. The substance is quite soluble in alcohol at room temperature, very readily in acetone, chloroform, or benzene. It is difficultly soluble in cold ligroin, readily on heating, and also dissolves sparingly in boiling water.

Subs., 0.1541: (Kjeldahl), 5.85 cc. 0.1 *N* HCl.

Calc. for  $C_{12}H_{16}O_3NCl$ : N, 5.44. Found: 5.32.





## A NEW APPARATUS FOR MEASURING SURFACE TENSION.

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Surface tension is probably one of the most difficult phenomena to measure. Although a great deal of ingenuity has been spent for almost a century in devising accurate techniques, the figures obtained deviate more from each other for the same substance, according to different authors, than any other constant characterizing the substance. It is well known that the two classes of methods of measurement, the static and the dynamic give entirely different results when applied to the same liquid. The following figures illustrate these differences.

	Dynamic.	Static.
Water.....	73	73
Sodium oleate.....	26	79
Heptylic acid.....	54	68

The static values were obtained by the capillary tube method, and the dynamic values by the oscillating jet method. Nevertheless, under given conditions, all the methods give practically concordant values in the case of pure liquids, but not in the case of solutions.

With the static methods (among which are weight of hanging drops, rise in a capillary tube, adhesion of a disc to the surface, excess of pressure in a spheric bubble produced in a liquid, direct measurement of curvature of the surface), the following figures are obtained for the surface tension of water.

	Dynes. cm.
Method of pulling off a ring.....	81.0
“ “ capillary tube (air).....	75.5
“ “ “ “ (water vapor).....	73.0

Some authors give 82 for the first method, others, 80, and for the third, some give 68.

Temperature plays an important part. It may be represented by the simple formula:

$$A = A_0 (1 - \alpha t)$$

The cleanliness of the apparatus containing the liquids and of the measuring devices is extremely important, for a minute trace of greasy substance spoils the results.

The importance of the action of surface tension in biological phenomena is well known, but all the techniques of measurement are either complicated (static methods: capillary undulations, drop rebounding, capillary jets, rebounding jets), or long (drop method), and it is desirable to have a simple apparatus by means of which the surface tension, and especially the variation of surface tension of a given liquid, could be readily measured, with sufficient accuracy. For this reason the apparatus to be described has been designed (Fig. 1). There is no new principle in it; it is based upon adherence of a ring, or of any other design, to the liquid (Weinberg). It is simply a torsion balance, but instead of measuring the tension by means of weights (which is time-consuming, and makes two readings necessary), the torsion of the wire is used to counteract the tension of the liquid film and to break it. A single reading on a dial indicating the degree of torsion of the wire gives a figure, which, if the apparatus has been previously standardized with water, gives the surface tension of the liquid by a simple proportion. According to the fact that the torsion of the wire for water, which has the highest surface tension, is only  $72^\circ$ , we can assume that, within these limits, the strain of the wire is proportional to the angle of torsion, so that no table of correction is needed (see Fig. 2). Of course, the apparatus may be used for standard measurements also, by simply forcing the lever to come back to its former position by means of weights after the tearing of the membrane has taken place. By using the comparison method, the figures are reliable and constant, and the time necessary to make one measurement does not exceed 15 to 30 seconds. A very small quantity of the liquid is required,—about 1 cc. in a watch-glass,—but the apparatus could be fixed in such a way that it would need only 0.5 cc.



FIG. 1.

The tension of the wire can be adjusted. The torsion is controlled by a gear, which moves very slowly. The dial shown in Fig. 1 is attached to the wire and turns with it. A fixed needle indicates the angle of torsion. The apparatus described is home-made, and would be greatly improved by using a large dial and a worm-gear for controlling the torsion.



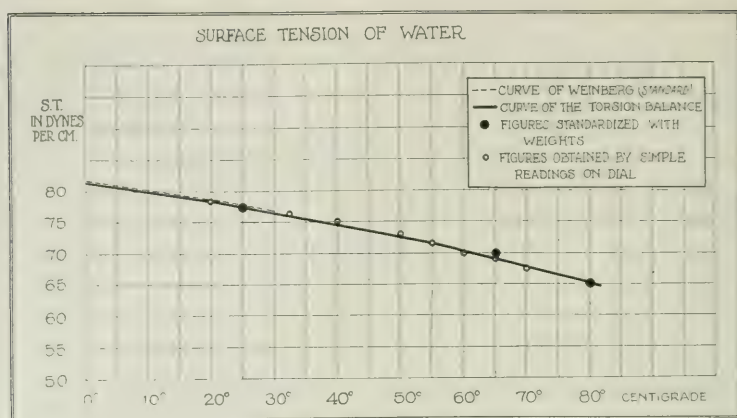


FIG. 2.

With the apparatus described above, with a platinum ring, the circumference of which is equal to 4 cm., 77 dynes per cm. were found for the surface tension of ordinary distilled water, at 25°C., 81 dynes at 0°, and 65 dynes at 80° (see Fig. 3).

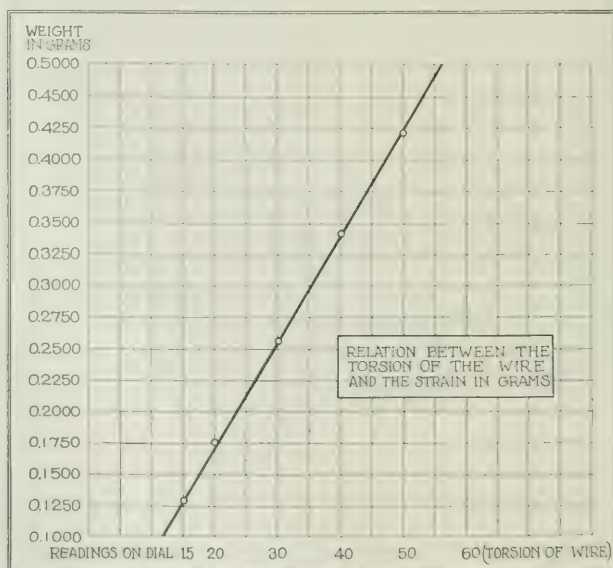


FIG. 3.

## AMPHOTERIC COLLOIDS.

### V. THE INFLUENCE OF THE VALENCY OF ANIONS UPON THE PHYSICAL PROPERTIES OF GELATIN.

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It was shown in preceding papers that while all gelatin salts with univalent cation show a comparatively high osmotic pressure, a high degree of swelling, a high viscosity, and a high alcohol number, the gelatin salts with bivalent metal (and trivalent metal) show for the same pH and gelatin concentration a low osmotic pressure, low degree of swelling, low viscosity, and a low alcohol number. Since the conductivities of the two types of gelatin salts were found to be practically the same, the valency effect could not be attributed to differences in the degree of ionization and "hydratation" of the gelatin ions. The writer suggested as a tentative explanation the assumption of an aggregate formation of a stoichiometrical character. He had shown that two gelatin ions combine with one bivalent cation and it was suggested that in the process of electrolytic dissociation these two gelatin ions form one anion with two charges. This suggestion is able to explain quantitatively the results actually observed.<sup>1</sup>

In one of his previous publications the writer had already called attention to the fact that bivalent anions differ in their valency effect from bivalent cations.<sup>2</sup> While  $\text{SO}_4$  has a similar depressing effect upon the physical properties of gelatin as Ca or Ba, the same does not seem to be true for other bivalent and trivalent anions like oxalate, succinate, tartrate, citrate, or phosphate. The acids of these latter anions seemed to act as if they were monobasic acids, one molecule of these acids being capable of combining with one gelatin ion only.

<sup>1</sup> Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 483.

<sup>2</sup> Loeb, J., *J. Biol. Chem.*, 1918, xxxiv, 489.

It seemed very important to test this possibility, since a decision might serve as a crucial test for or against our purely chemical conception of the behavior of colloids, according to which the effect of an electrolyte upon the physical properties of the colloid is due to the formation of real chemical compounds between the colloid and one of the ions of the electrolyte. If it can be shown that  $\text{SO}_4$  has a similar depressing effect as a bivalent cation we must also be able to prove that  $\text{SO}_4$  combines with two molecules of gelatin; and if it can be shown that  $\text{PO}_4$ , citrate, oxalate, succinate, etc., have no such depressing effect upon the physical properties of gelatin but behave like Br, Cl,  $\text{NO}_3$ , and acetate, we must also be able to prove that one molecule of each of these acids combines with only one molecule of gelatin.

We are able to decide this question in favor of the purely chemical theory of colloids.

The decision was rendered possible through the proof that we can ascertain the amount of acid in combination with a given mass of gelatin by titration with  $\text{NaOH}$ . If we treat gelatin with acid and titrate afterwards with  $\text{NaOH}$ , we obtain the mass of acid in combination with the gelatin with the aid of two corrections; namely, first by deducting from the titration value a constant value which is 1.8 cc. 0.01 N  $\text{NaOH}$  for 10 cc. of a 1 per cent gelatin solution, titrating to neutrality ( $\text{pH} = 7.0$ ). This constant value 1.8 cc. 0.01 N  $\text{NaOH}$  is the quantity of alkali required to bring 0.1 gm. isoelectric gelatin to  $\text{pH} 7.0$ . The correctness of this method was demonstrated in the case of  $\text{HBr}$  and  $\text{HCl}$  in which it was possible to compare the amount of  $\text{NaOH}$  required for the titration of a 1 per cent gelatin solution with the results of volumetric analysis for Br according to Volhard's methods. It was found that the Br (or Cl) numbers agreed with the  $\text{NaOH}$  numbers when the constant value mentioned above was deducted. In that paper<sup>3</sup> we had titrated for  $\text{pH} = 9.0$ , while in the experiments reported in the present paper we titrate for  $\text{pH} = 7.0$ ; as a consequence the value of the constant given in the first paper was higher than 1.8 cc. 0.01 N  $\text{NaOH}$ .

<sup>3</sup> Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 363.

In addition a second correction is needed as explained in a previous paper. The gelatin treated with acid contains some free acid, the titration value of which has also to be deducted from the titration number. This correction can be found by titration of the same acid at the same pH but free from gelatin.

With the aid of these two corrections we are also able to investigate the effect of acid on gelatin in the presence of the acid. It was observed that the maximal osmotic pressure of gelatin chloride or sulfate is reached at a pH of between 3.3 to 3.5. This maximum is never reached when we wash the gelatin four or six times with  $H_2O$  after a treatment with an acid of moderate concentration, as was done in our previous experiments, since in this washing the acid formed by hydrolytic dissociation is removed, which causes new hydrolysis and in this way in successive washings a rapid decline of the pH results, as will be shown more definitely in a subsequent paper.

Since it was necessary for our purpose to be always sure of ascertaining the maximal osmotic pressure a 1 per cent solution of isoelectric gelatin can reach when treated with different acids, we were forced to adopt a different method from that described in the preceding papers. We melted 1 gm. of gelatin rendered isoelectric in the manner described in a previous paper and then added different quantities of 0.01 N or 0.1 N acid and made up the volume to 100 cc.<sup>4</sup> This solution was put into a collodion bag of a capacity of about 50 cc. and the bag was put into a beaker containing 400 cc. of the same percentage of acid in distilled water as that added to the gelatin solution. The collodion bag was closed tightly with a perforated rubber stopper containing a glass tube serving as a manometer to measure the osmotic pressure of the gelatin solution. After about 18 hours, when osmotic equilibrium was reached, the pH, the conductivities, and the titration numbers of the acid inside and outside the bag were determined, as were also the osmotic pressures.

<sup>4</sup> For the sake of briefness we shall designate such solutions as 1 per cent gelatin solutions, although this does not take into account the increase in weight of the gelatin due to the combination with another ion.



Table I gives the data of one experiment with a 1 per cent solution of isoelectric gelatin made up in 100 cc. of  $H_2O$  containing varying quantities of 0.01 N HBr, stated in the upper horizontal row. The second horizontal row of figures gives the pH of the gelatin solution at the end of the experiment, and the third row the figures for the cc. of 0.01 N Br found by Volhard's method, in 10 cc. of the gelatin solution. These latter figures are too high since they include the free HBr in the solution. We can find the value for the free HBr solution from the pH and this value must be deducted from the titration number for Br. This deduction gives us the corrected Br values of the horizontal Row 4. Row 5 gives the number of cc. of 0.01 N HBr found in 10 cc. of the gelatin solution by titrating with NaOH to pH = 7.0. This value demands, as stated, two corrections; first, a deduction of the amount of NaOH needed to bring 10 cc. of 1 per cent isoelectric gelatin solution to pH = 7.0. This value is 1.8 cc. of 0.01 N NaOH for 10 cc. of a 1 per cent solution of gelatin. The second correction is that for the free HBr present in the gelatin solution which can be measured by titrating free HBr of the same pH as that of the gelatin solution to pH = 7.0. Row 6 gives the titration values for NaOH after the two corrections have been made. These corrected figures should give us the amount of HBr in combination with 0.1 gm. of gelatin. The reader will notice that the corrected numbers for the direct titration for Br after Volhard (Row 4), and the corrected numbers for titration with NaOH (Row 6), are, within the limits of the accuracy of our method, identical, as they should be. This identity is of the greatest importance for the subject we intend to discuss in this paper, since in the case of other acids (with the exception of HCl and  $H_3PO_4$ ) we can ascertain the amount of acid in combination with 0.1 gm. of gelatin only indirectly by titrating with NaOH. It was, therefore, necessary for us to be sure that this titration gives us the correct amount of acid in combination with 0.1 gm. of gelatin if we make the two corrections mentioned, and the identity of the titration numbers by the direct Volhard method for Br and by the indirect method of titrating with NaOH gives us this certainty.

Row 7 gives the osmotic pressure in mm. of the height of a column of the 1 per cent gelatin solution. Row 8 gives us the conductivity

TABLE I.

1 per cent solution of isoelectric gelatin made up in 100 cc. H <sub>2</sub> O containing varying quantities of 0.01 N HBr.																
	50	40	35	30	25	22.5	20	17.5	15	12.5	10	7.5	5	2.5	1	0.5
1. Cc. 0.01 N HBr added.....																
2. pH of gelatin solution.....	2.8	3.0	3.1	3.15	3.25	3.3	3.35	3.4	3.5	3.6	3.75	3.9	4.1	4.3	4.5	4.7
3. Cc. 0.01 N Br found in 10 cc. of gelatin solution.....	8.55	7.3	6.8	6.4	5.7	5.4	4.85	4.45	4.0	3.3	3.0	2.4	1.5	0.65	0.2	0.1
4. Corrected values of Br.....	7.0	6.1	5.8	5.6	5.1	4.9	4.5	4.05	3.66	3.05	2.8	2.28	1.42	0.55	0.15	0
5. Cc. 0.01 N NaOH required to bring 10 cc. of gelatin solution to pH 7.0.....	10.0	9.2	8.4	8.0	7.3	7.0	6.55	6.3	5.8	5.15	4.7	4.2	3.5	2.7	2.15	1.9
6. Corrected NaOH values.....	6.7	6.25	5.6	5.4	4.9	4.7	4.35	4.1	3.66	3.1	2.7	2.28	1.62	0.8	0.3	0
7. Osmotic pressure in mm. of height of column of a 1 per cent gelatin solution.....	188	230	256	292	294	310	306	330	330	305	292	256	204	100	45	26
8. Conductivity ( $\frac{10,000}{\text{ohms}}$ ), corrected for pH.....		21.2	18.85	16.8	14.9		13.1	11.1	9.7		7.2			1.47	0.51	
Measurements of pure acid solution (free from gelatin) in the outside beaker.																
9. pH of acid solution.....	2.2	2.3	2.35	2.4	2.5	2.6	2.75	2.9	3.0	3.1	3.15	3.25	3.35	3.8	4.3	4.5
10. $\beta$ number.....	4.85	3.6	3.15	2.7	2.3	1.9	1.7	1.5	1.2	1.0	0.8	0.6	0.35	0.2	0.15	0.05
11. NaOH number.....	4.7	3.6	3.0	2.6	2.1	1.9	1.65	1.45	1.15	1.0	0.8	0.6	0.4	0.2	0.1	0.05
12. Conductivity ( $\frac{10,000}{\text{ohms}}$ ).....	54.6	42.8	37.0	32.8	25.6	22.3	20.0	17.1	13.8	11.35	9.0	6.67	4.07	1.86	0.86	0.48

$\left(\frac{10,000}{\text{ohms}}\right)$  corrected for the pH (*i.e.*, after deduction of the conductivity of a free acid solution with the pH found in the gelatin solution).

Rows 9 to 12 give the measurements of the pure acid solution (free from gelatin) surrounding the collodion bag containing the gelatin solution. The pH in the outside solution is always lower than inside the bag and this difference may find its explanation on the basis of the theory of equilibrium developed by Donnan for such cases. The values for titration for Br by the Volhard method and for the titration for HBr by titration with NaOH are practically identical for this pure acid solution, thus showing that the degree of accuracy of the method of titration was adequate.

In Table II the measurements for 1 per cent isoelectric gelatin treated with  $\text{H}_2\text{SO}_4$  are given. Here we can ascertain the amount of  $\text{SO}_4$  in combination with 0.1 gm. of gelatin only indirectly by titrating with NaOH, applying the two corrections mentioned. All the other measurements are the same as in the experiment with HBr.

A comparison of the corrected NaOH values in Row 4, Table II, with those in Row 6, Table I, shows that they are practically identical for the same pH. Thus for pH = 3.35 the corrected NaOH value is 4.4 in the case of gelatin sulfate, and 4.35 for gelatin bromide. Since the experiments with HBr have shown that these corrected NaOH values are identical with the Br values we can state, that for a given pH 1 gm. of gelatin is in combination with twice as many atoms of Br as with radicles of  $\text{SO}_4$ ; or in other words, that  $\text{H}_2\text{SO}_4$  behaves toward gelatin as a dibasic acid. Fig. 1 illustrates this identity of the curves for NaOH values of the two acids. In spite of the identity of equivalents of these two ions in combination with 1 gm. of gelatin the osmotic pressures are very different for the two types of gelatin salts. This is illustrated by Fig. 2, where the ordinates represent the osmotic pressures and the pH are the abscissæ. It is obvious that the values of the osmotic pressure of gelatin bromide are considerably higher than the values for the osmotic pressure of gelatin sulfate. The reader will notice that the maximum osmotic pressure for gelatin bromide is about 325 mm., which is identical with the maximal osmotic pressure found for gelatin salts with univalent cation, *e.g.* sodium gelatinates; while the maximum value for gelatin sulfate is about 130, almost identical with the maximum for calcium

TABLE II.

1 per cent solution of isoelectric gelatin made up in 100 cc. H <sub>2</sub> O containing varying quantities of m/100 H <sub>2</sub> SO <sub>4</sub> .																
1. Cc. m/100 H <sub>2</sub> SO <sub>4</sub> .....	45	40	35	30	25	22.5	20	17.5	15	12.5	10	7.5	5	2.5	0	
2. pH of gelatin solution.....	2.4	2.45	2.5	2.65	2.75	2.85	2.9	2.95	3.0	3.1	3.2	3.35	3.55	4.1	4.8	
3. Cc. 0.01 N NaOH required to bring 10 cc. of gelatin solution to pH 7.0.....	15.2	14.4	13.8	12.5	11.6	10.9	10.0	10.4	9.1	8.3	7.8	6.6	5.5	3.8	1.8	
4. Corrected NaOH values.....	9.55	9.2	9.0	8.9	8.2		6.7		6.2	5.7	5.4	4.4	3.4	1.85	0	
5. Osmotic pressure in mm. of height of column of a 1 per cent gelatin solution.....	63	74	77	80	87	90	91	92	114	123	129	132		112	34	
6. Conductivity $\left(\frac{10,000}{\text{ohms}}\right)$ , corrected for pH.....	31.0		30.1		26.0		18.1		15.25	10.85		9.8	7.3	3.95	0.52	
Measurements of pure acid solution (free from gelatin) in the outside beaker.																
7. pH of acid solution.....	2.1	2.2	2.2	2.3	2.35	2.4	2.45	2.5	2.55	2.65	2.75	3.0	3.3	3.5	5.0	
8. NaOH number.....	8.0	7.3	6.4	5.2	4.4	3.85	3.4	3.0	2.4	1.8	1.4	1.1	0.7	0.3		
9. Conductivity $\left(\frac{10,000}{\text{ohms}}\right)$ .....	81.3	74.0	65.8	55.3	47.6	47.9	35.5	33.2	27.4	21.4	16.1	11.75	7.46	3.22	0.22	



gelatinate. The maxima for both gelatin bromide and gelatin sulfate are found at the same pH, namely between 3.3 and 3.5. van't Hoff's theory would ascribe the difference in the maximal osmotic pressures of the two acids to a corresponding difference in the number of particles in solution; while the colloid chemists would ascribe the difference in osmotic pressure to a difference in the "hydration" due to a difference in the ionization of the two gelatin salts.

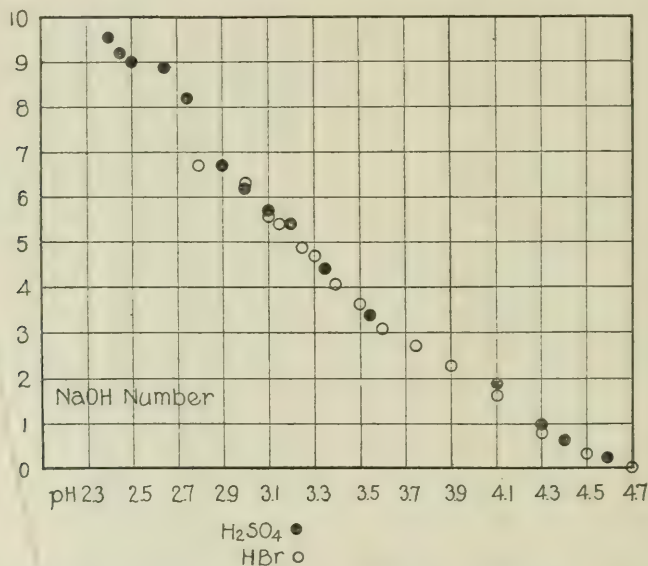


FIG. 1. Relative amount of HBr and  $\text{H}_2\text{SO}_4$  in combination with gelatin. Abscissæ represent pH; ordinates, amount of 0.01 N HBr and  $\text{H}_2\text{SO}_4$  bound by 0.1 gm. of gelatin. The identity of the curves for the two acids proves that  $\text{H}_2\text{SO}_4$  combines with gelatin as a dibasic acid.

The curves for conductivity of the two gelatin salts (plotted according to the corrected conductivity values of Table I and Table II) differ but slightly (Fig. 3). As a matter of fact, if other experiments with the two acids are taken into account (one of which will be given later the curves for the conductivities of the two gelatin salts are practically identical if plotted over pH as abscissæ. This identity of the conductivity curves contradicts the hydration hypothesis of the colloid chemists.

We therefore are confronted with the same situation as in the case of sodium gelatinate and calcium gelatinate, where we also found practically equal conductivity combined with a ratio of osmotic pressures of 3:1 for the two metal gelatinates mentioned. In the latter case we offered the tentative assumption that the Ca ion combines

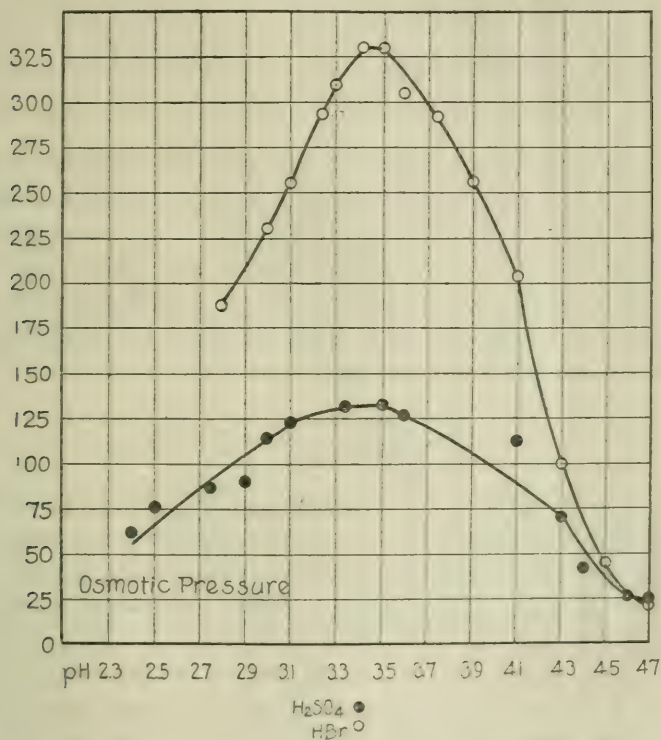


FIG. 2. Osmotic pressure curves for gelatin sulfate and gelatin bromide. Abscissæ represent pH; ordinates, osmotic pressure, showing that for the same pH the osmotic pressure is higher when  $HBr$  than when  $H_2SO_4$  is added to gelatin. The maximal osmotic pressure for both acids is found at the same pH (about 3.5).

with two gelatin anions, these two gelatin anions remaining together in a single aggregate when electrolytic dissociation occurs. If the compound  $Ca_2$  gelatin, dissociates into two Ca ions and one aggregate of four gelatin anions carrying four charges, the same number of charges, namely eight, would be carried by the dissociation of four

sodium gelatinate molecules yielding eight charges and eight ions. This would produce equality of charges and a ratio of osmotic pressures of 3:8. The same assumption applied to gelatin bromide and gelatin sulfate would demand that  $\text{gelatin}_4(\text{SO}_4)_2$  dissociate into three particles, two negative  $\text{SO}_4$  ions and one aggregate ( $\text{gelatin}_4$ ) with four positive charges; while in the case of gelatin bromide the same number of charges would be carried by eight separate ions, four Br and four separate positive gelatin ions. This would again produce equality in the number of charges and a ratio of 3:8 in the number of particles.

When pH becomes less than 3.3 the values for osmotic pressure drop again. This drop which had been noticed by previous investigators, especially by Pauli, is ascribed by the latter to a diminution in the degree of electrolytic dissociation of the gelatin salt. This assumption meets, however, with a difficulty in the fact that the conductivity of the gelatin salts does not show this drop but continues to rise steeply for values of pH less than 3.3.

*Antagonism between HBr and  $\text{H}_2\text{SO}_4$ .*

The real crux between the colloidal and the chemical conception seems to lie in the question of the justification of the assumption of "hydration" as the cause of osmotic pressure of protein solutions. The writer does not question the possible correctness of the idea that ions are in general surrounded by a jacket of water molecules; he only doubts the correctness of the idea that this possible hydration of the protein ion is the cause of the osmotic pressure, the swelling, and the other physical properties of proteins as Pauli, Michaelis, and others assume. The following experiment seems to be a further proof against this application of the hydration hypothesis. We had seen that a 1 per cent solution of both gelatin bromide and gelatin sulfate reaches the maximal osmotic pressure at a pH of about 3.5 and that for this pH the same amount of gelatin is combined with equivalent amounts of Br and  $\text{SO}_4$ , namely 0.1 gm. of gelatin binding about 3.6 cc. of 0.01 N Br or  $\text{SO}_4$ . Both solutions have the same conductivity, namely about 9.7. In order to produce gelatin bromide or gelatin sulfate of a pH of about 3.4 it is necessary

that 1 gm. of isoelectric gelatin should be melted and added to 15 cc. of 0.01 N HBr or  $\text{H}_2\text{SO}_4$  and that the volume be made up to 100 cc. by adding distilled water. By mixing HBr and  $\text{H}_2\text{SO}_4$  in various

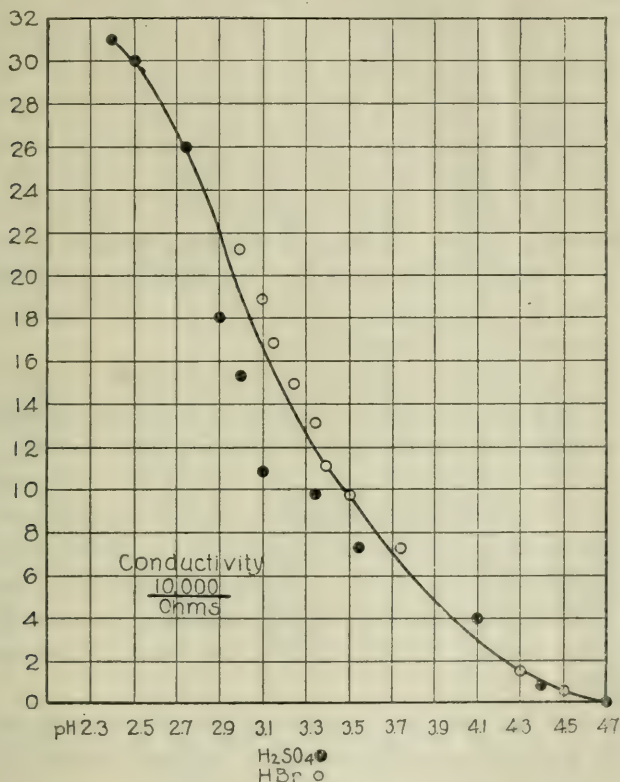


FIG. 3. Conductivity curves for gelatin sulfate and gelatin bromide. Abscissæ represent pH; ordinates, conductivity of gelatin sulfate and gelatin bromide, showing that aside from a few irregular values the curves are practically identical for the two acids. This disproves the assumption that the differences in osmotic pressure, as shown in Fig. 2, can be attributed to differences in ionization and "hydration" of the two gelatin salts.

proportions but always adding 15 cc. of 0.01 N acid to 1 gm. of isoelectric gelatin, melting the latter, and making up the volume to 100 cc. by the addition of water, the amount of gelatin salt formed and its degree of ionization, *i.e.* the conductivity, should remain the



same, while the osmotic pressure should vary according to the relative proportion of HBr and  $\text{H}_2\text{SO}_4$  used. HCl was used in this experiment instead of HBr since we had found that HCl and HBr act qualitatively and quantitatively alike on the physical properties of gelatin. The mixtures in which the 15 cc. of 0.01 N acid were prepared were made up as follows: 15 cc. 0.01 N HCl + 0 cc. 0.01 N  $\text{H}_2\text{SO}_4$ ; 14 cc. 0.01 N HCl + 1 cc. 0.01 N  $\text{H}_2\text{SO}_4$ ; 13 cc. 0.01 N HCl + 2 cc. 0.01 N  $\text{H}_2\text{SO}_4$ , etc., and finally 0 cc. 0.01 N HCl + 15 cc. 0.01 N  $\text{H}_2\text{SO}_4$ . Table III gives the numerical values for the pH, the corrected titration number, the conductivity, and the osmotic pressure of each gelatin solution. While the titration number (Row 2) and the values for conductivity (Row 3) are everywhere practically identical, the osmotic pressures vary from 300 to 128. Fig. 4 gives the graphical expression of the result.

It is obvious that the values for osmotic pressure are not the algebraic mean between the values for pure HCl and pure  $\text{H}_2\text{SO}_4$ . The curve is convex towards the axis of abscissæ, thus showing that the depressing effect of  $\text{SO}_4$  increases more rapidly than the quantity of  $\text{SO}_4$  added. This is characteristic for a true antagonistic salt effect.

The main result is unequivocal; namely, that the differences in the osmotic pressures are not due to differences in ionization and hydration of protein ions as the colloid chemists assume, since the conductivities remain constant while the osmotic pressures vary considerably.

What is true for the relative influence of the two acids on osmotic pressure is also true for their relative influence on swelling, and viscosity of gelatin as is shown in Figs. 5 and 6.

*Proof that Dibasic and Tribasic Acids (with the Exception of  $\text{H}_2\text{SO}_4$ ) Behave Practically like Monobasic Acids toward Gelatin.*

Of the other dibasic and tribasic acids which the writer has thus far investigated, namely oxalic, tartaric, succinic, citric, and phosphoric acid, none shows the valency influence. We are able to give the explanation for this peculiar behavior by proving that all these acids react with gelatin practically as if they were monobasic acids.

TABLE III.

1 per cent solution of isoelectric gelatin made up in 100 cc. H <sub>2</sub> O containing varying quantities of 0.01 N HCl and H <sub>2</sub> SO <sub>4</sub> .																			
	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	0			
Cc. 0.01 N HCl.....	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15			
Cc. 0.01 N H <sub>2</sub> SO <sub>4</sub> .....																			
1. pH of gelatin solution.....	3.4	3.35	3.4	3.35	3.35	3.4	3.35	3.4	3.4	3.35	3.35	3.35	3.35	3.35	3.35	3.35			
2. Corrected NaOH values.....	3.3	3.6	3.6	3.6	3.3	3.5	3.8	3.9	3.7	3.9	3.9	3.8	4.0	4.1	4.1	4.1			
3. Conductivity $\left(\frac{10,000}{\text{ohms}}\right)$ , corrected for pH.....	9.2	10.4	9.1	9.5	8.4	8.1	9.2	9.2	9.5	9.1	9.5	9.3	9.6	9.3	9.4	9.2			
4. Osmotic pressure in mm. of height of column of a 1 per cent gela- tin solution.....	300	282	254	236	210	205	172	185	162	166	151	148	136	134	127	128			

We repeated the experiments represented in Tables I and II with a number of other acids, the method of adding acid to gelatin being the same in all cases, though the quantity of acid needed to produce

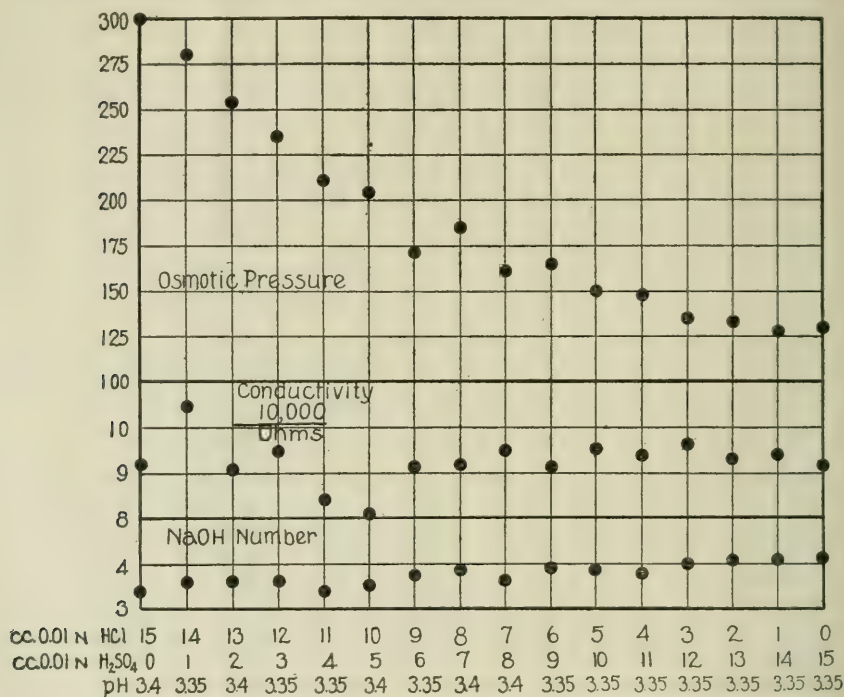


FIG. 4. Effect of mixtures of HCl and H<sub>2</sub>SO<sub>4</sub> upon conductivity and osmotic pressure of gelatin. Abscissæ represent mixtures of the two acids; ordinates of lower curve, amount of acid in combination with 0.1 gm. of gelatin. The curve is a straight line, proving that equivalent amounts of H<sub>2</sub>SO<sub>4</sub> and HCl are bound by 0.1 gm. of gelatin. Ordinates of middle curve, corrected conductivities of gelatin treated with different proportions of a mixture of the two acids. The curve is also practically a straight line. Upper curve, osmotic pressure curve, showing that the osmotic pressure is the greater the greater the proportion of HCl is in the mixture. The curve is convex to the axis of abscissæ, which is characteristic for antagonism. Notice identity of conductivities and the difference in osmotic pressure, contradicting the hydration theory of colloid chemists.

the same pH was different for different acids. In the case of phosphoric acid we determined directly (with the uranylacetate method) the PO<sub>4</sub> in combination with 10 cc. of a 1 per cent gelatin solution

treated with different quantities of  $\text{H}_3\text{PO}_4$ . In the case of  $\text{HNO}_3$  and oxalic acid we determined the amount of acid in combination with the same amount of gelatin by titration with  $\text{NaOH}$  for  $\text{pH} = 7.0$ ,

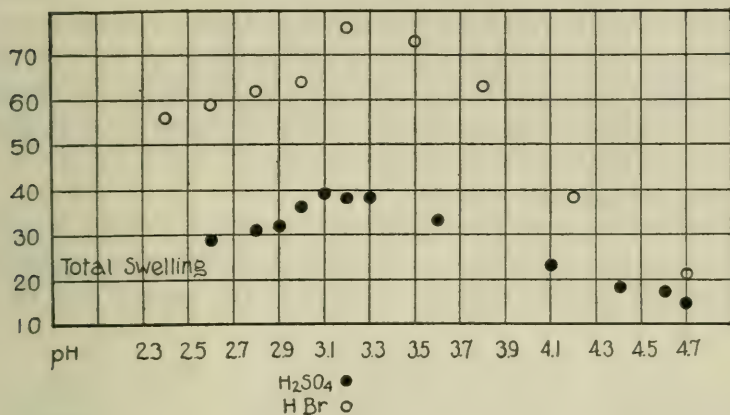


FIG. 5. Relative influence of  $\text{HBr}$  and  $\text{H}_2\text{SO}_4$  upon swelling.

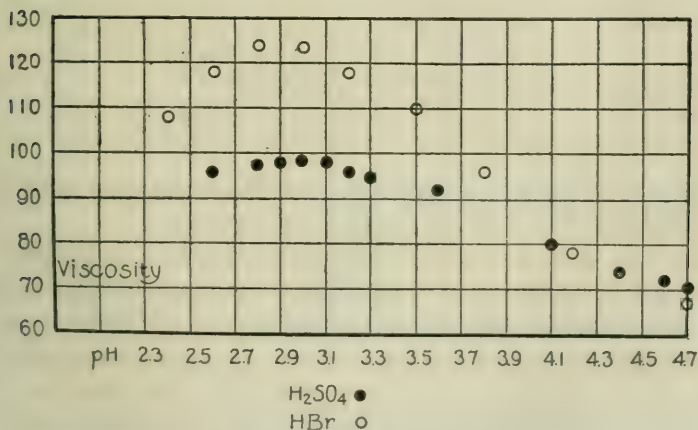


FIG. 6. Relative influence of  $\text{HBr}$  and  $\text{H}_2\text{SO}_4$  upon viscosity.

and making the two corrections discussed before. Table IV gives the equivalents of these three acids in combination with 0.1 gm. of gelatin for different pH in terms of cc. of 0.01 N  $\text{NaOH}$  used for titration.



The values found for  $\text{HNO}_3$  are slightly less than those found for  $\text{HBr}$  and  $\text{HCl}$ . This harmonizes with the fact that the effect of  $\text{HNO}_3$  on the physical properties of gelatin is also slightly less than the effect of  $\text{HCl}$  or  $\text{HBr}$  for the same pH. A comparison of the figures for  $\text{NaOH}$  values for  $\text{HNO}_3$  and for the  $\text{PO}_4$  values, found by titration for  $\text{PO}_4$  (Table IV, Rows 1 and 3) shows for the two values practically the ratio of 1:3 at the same pH; *i.e.*, three times as much  $\text{H}_3\text{PO}_4$  as  $\text{HNO}_3$  is in combination with the same mass of gelatin. The figures for  $\text{HNO}_3$  and oxalic acid (Rows 1 and 2, Table IV) give the ratio of approximately 1:2.

We therefore see in these figures the proof that actually one molecule each of phosphoric and oxalic acids is in combination with only one molecule of gelatin; and we see in this the explanation for the

TABLE IV.

Cc. of 0.01 N acid in combination with 10 cc. of a 1 per cent gelatin solution at different pH.										
pH	3.1	3.2	3.3	3.4	3.5	3.7	3.9	4.1	4.2	4.3
1. $\text{HNO}_3$ .....	4.35	4.1	3.6	3.2	2.85	2.45	1.9	1.45		0.75
2. Oxalic acid.....	9.6	8.75	7.6	6.7	6.00	4.3	3.0		1.65	
3. $\text{H}_3\text{PO}_4$ .....		12.4	10.4	9.8	9.00	7.4	5.8	4.5	2.6	2.1

fact that they do not depress the osmotic pressure as does  $\text{H}_2\text{SO}_4$ , which behaves like a dibasic acid towards gelatin.

What has been proved for oxalic and phosphoric acids holds also for tartaric, succinic, and citric acids. These latter two acids are very weak and thus relatively large quantities of acid have to be added to bring the solution to the desired pH. The same is true for acetic acid. The equilibrium conditions seem to be such that a very large amount of undissociated acid is found inside the collodion bag and it seems for the present impossible to determine by titration with  $\text{NaOH}$  the amount of these acids in actual combination with gelatin.

The fact that all the dibasic and tribasic acids mentioned (with the exception of  $\text{H}_2\text{SO}_4$ ) behave towards gelatin practically like monobasic acids is a further proof for the theory that the influence of acids on gelatin is determined by the relative amount of gelatin salt formed

through the addition of acid by gelatin. If  $\text{H}_3\text{PO}_4$  behaves towards gelatin like a monobasic acid the curves for osmotic pressure for gelatin phosphate should be identical with the curves for gelatin nitrate when plotted over pH as abscissæ. Fig. 7 shows that this is actually the case. The maximum for the two curves lies between pH 3.3 and 3.5 and is about 300 mm. Fig. 8 shows that the osmotic pressure curves for gelatin acetate and citrate are also identical and

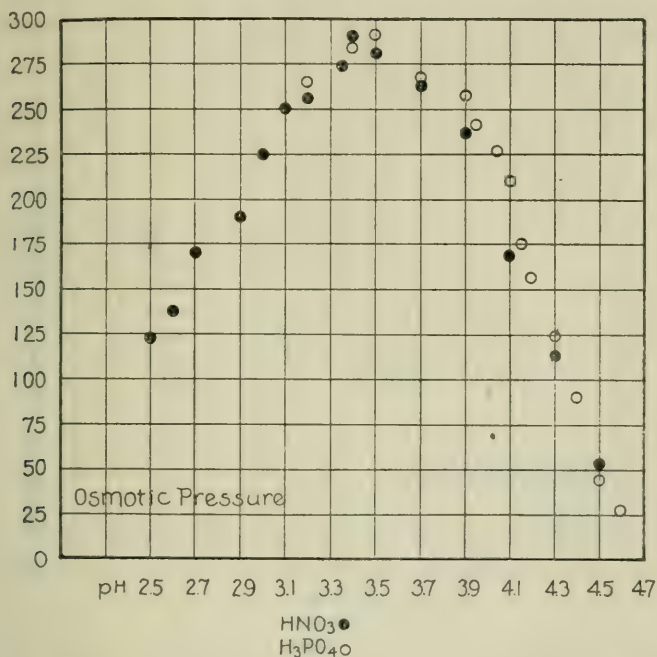


FIG. 7. Showing identity of influence of  $\text{HNO}_3$  and  $\text{H}_3\text{PO}_4$  upon osmotic pressure of gelatin solution for the same pH.

that they agree with the curves for gelatin nitrate and gelatin phosphate as well as with those for gelatin bromide (Fig. 1).

What is true for the osmotic pressure curves is also true for the curves for the other physical properties of gelatin. Thus Fig. 9 gives the curves for viscosity of 1 per cent solutions of gelatin bromide, oxalate, and tartrate, showing that they are practically identical when plotted with pH as abscissæ. Fig. 10 gives the curves

for swelling for gelatin oxalate, and chloride, which are also approximately identical.<sup>5</sup> The fact that the curves for viscosity and swelling are similar to those for osmotic pressure has been pointed out so often in previous papers that we need not dwell further on this point.

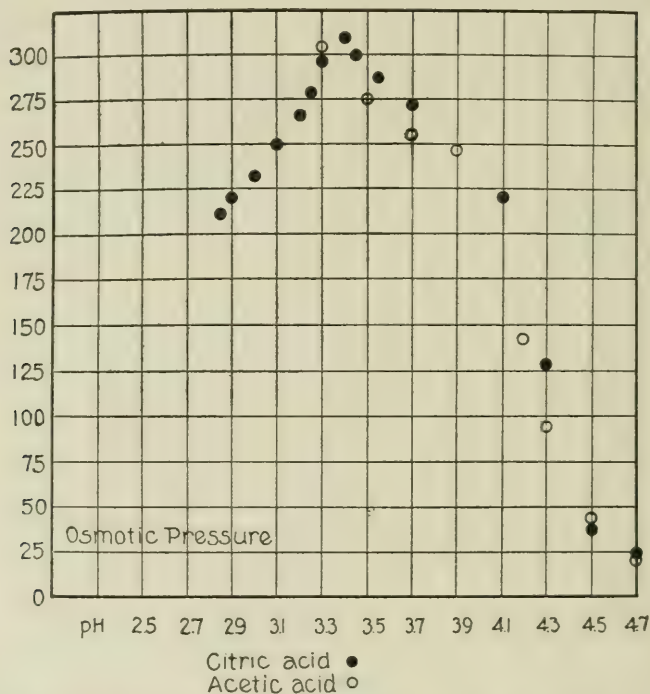


FIG. 8. Showing identity of influence of citric and acetic acids upon osmotic pressure of gelatin solution for the same pH. Curves in Fig. 8 are identical with those in Fig. 7.

<sup>5</sup> The close proximity of the effects of different acids does not harmonize with the contention of the colloid chemists who report a typical difference in the effects of the acids according to the nature of the anion. This is an error due to the fact that they failed to determine the pH of their solutions and to compare the behavior of gelatin solutions of the same pH, comparing instead the effects of equimolecular concentrations of different acids, without making any of the necessary corrections discussed in this paper.

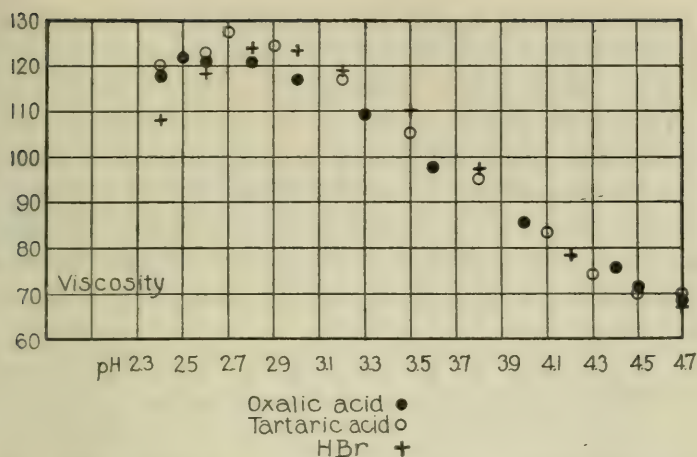


FIG. 9. Viscosity curves of gelatin treated with HBr, tartaric, and oxalic acids are identical when plotted over pH as abscissæ.

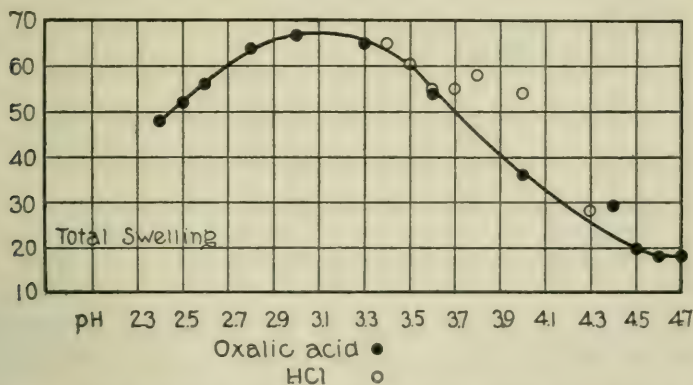


FIG. 10. Curves showing the influence of HCl and oxalic acid upon swelling of gelatin. The curves are identical when plotted over pH.

#### *Antagonism Experiments between HCl and Phosphoric and Oxalic Acids.*

It was to be expected that when  $\text{H}_3\text{PO}_4$  or oxalic acid is added to HCl or HBr it can produce no or only a slight antagonistic effect. The results of actual experiments support this expectation. The same experiment as that with HCl and  $\text{H}_2\text{SO}_4$  (represented in Fig. 4) was made by mixing different proportions of HCl and  $\text{H}_3\text{PO}_4$ .



15 cc. 0.01 N HCl + 0 cc. 0.01 N  $\text{H}_3\text{PO}_4$ ; 13.5 cc. 0.01 N HCl + 4.0 cc. 0.01 N  $\text{H}_3\text{PO}_4$ ; 12 cc. 0.01 N HCl + 8 cc. 0.01 N  $\text{H}_3\text{PO}_4$  to 3 cc. 0.01 N HCl + 32 cc. 0.01 N  $\text{H}_3\text{PO}_4$  and 0 cc. 0.01 N HCl + 40 cc. 0.01 N  $\text{H}_3\text{PO}_4$  were added each to 1 gm. of isoelectric gelatin and the solution was made up to 100 cc., the osmotic pressure of which was determined against 400 cc. of a pure acid solution of the same constitution and concentration as that added to gelatin. The pH was the same in all gelatin solutions, namely about 3.5, and the osmotic pressure was also the same, about 300 mm. (Fig. 11).

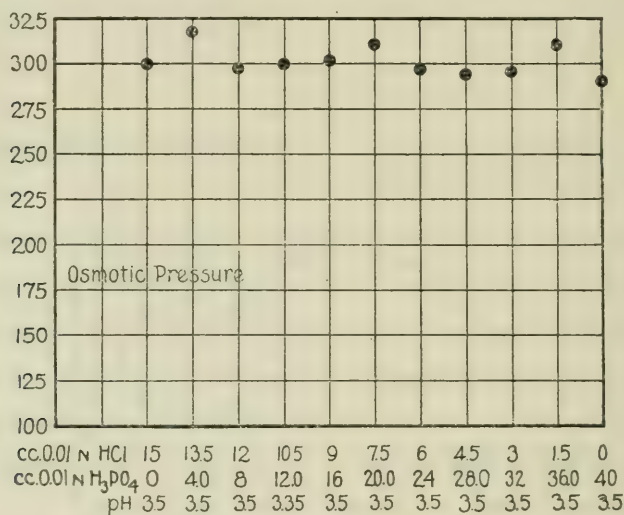


FIG. 11. Showing that  $\text{H}_3\text{PO}_4$  has no antagonistic effect on the influence of HCl upon the osmotic pressure of gelatin. The curve of osmotic pressure of different mixtures of the two acids for the same pH is a straight line.

There is a slight antagonistic effect when we mix HCl and oxalic acid (Fig. 12). This is due to the fact that the amount of oxalic acid in combination with a given mass of gelatin is less than twice the amount of HCl (or HBr) in combination with the same mass of gelatin. It is possible that a small fraction of the oxalic acid acts like a dibasic acid on gelatin while the greater part acts like a monobasic acid.

The fact that the dibasic and tribasic anions which have no or only a slight antagonistic effect act like monobasic acids towards gelatin harmonizes with the hypothesis of aggregation. Our idea of aggregation is a stoichiometrical one, making the number of gelatin ions forming one aggregate of gelatin ions a simple multiple of the valency number of the polyvalent ion with which they are in combination. Since phosphoric, citric, tartaric, succinic, and practically also oxalic acids behave like monobasic acids, *i.e.* since they can bind only one gelatin molecule, they cannot be expected to cause any aggregate formation and hence cannot produce any antagonistic salt action.

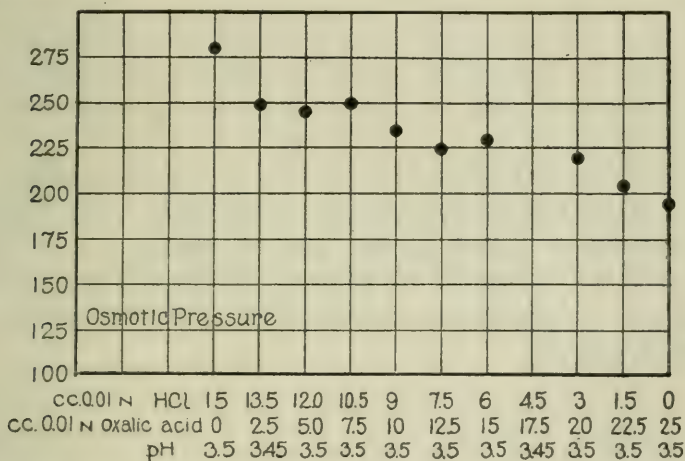


FIG. 12. Showing that oxalic acid has practically no antagonistic effect on the influence of HCl upon the osmotic pressure of gelatin.

#### SUMMARY.

1. When we plot the values of osmotic pressure, swelling, and viscosity of gelatin solutions as ordinates over the pH as abscissæ, practically identical curves are obtained for the effect of monobasic acids (HCl, HBr,  $\text{HNO}_3$ , and acetic acid) on these properties.

2. The curves obtained for the effect of  $\text{H}_2\text{SO}_4$  on gelatin are much lower than those obtained for the effect of monobasic acids, the ratio of maximal osmotic pressures of a 1 per cent solution of gelatin sulfate

and gelatin bromide being about 3:8. The same ratio had been found for the ratio of maximal osmotic pressures of calcium and sodium gelatinates.

3. The curves representing the influence of other dibasic and tri-basic acids, *viz.* oxalic, tartaric, succinic, citric, and phosphoric, upon gelatin are almost identical with those representing the effect of monobasic acids.

4. The facts mentioned under (2) and (3) permit us to decide between a purely chemical and a colloidal explanation of the influence of acids on the physical properties of gelatin. In the former case we should be able to prove, first, that twice as many molecules of HBr as of  $\text{H}_2\text{SO}_4$  combine with a given mass of gelatin; and, second, that the same number of molecules of phosphoric, citric, oxalic, tartaric, and succinic acids as of  $\text{HNO}_3$  or HCl combine with the same mass of gelatin. It is shown in the present paper that this is actually the case.

5. It is shown that gelatin sulfate and gelatin bromide solutions of the same pH have practically the same conductivity. This disproves the assumption of colloid chemists that the difference in the effect of bromides and sulfates on the physical properties of gelatin is due to a different ionizing and hydrating effect of the two acids upon the protein molecule.

## THE EFFECT OF VARIOUS ACIDS ON THE DIGESTION OF PROTEINS BY PEPSIN.

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(Received for publication, May 21, 1919.)

### I.

The widespread occurrence of antagonistic salt action on living tissues raises the question whether the underlying cause of the phenomenon might not be found in the action of the salts on the activity of the enzymes. A similar effect has been described by Falk<sup>1</sup> in the case of lipase. As Loeb<sup>2,3</sup> has shown, it is also possible to demonstrate antagonistic salt action on the physical properties of a protein; *i.e.*, gelatin. It seemed important therefore to determine whether or not such an effect was to be found in the influence of various acids on the digestion of proteins by pepsin.

The relative action of the various acids on the pepsin digestion of proteins has already been the subject of many investigations.<sup>4</sup> The literature on the subject is confused and contradictory, however, due largely to the fact that in the early work the effect of the hydrogen ion concentration was not taken into account and that the acids were usually compared in equimolecular or even equipercantage strengths. Attempts were made by Berg and Gies<sup>5</sup> to rule out this disturbing factor by using solutions containing equal calculated amounts of hydrogen ion. They took no account, however, of the "buffer" action of the weaker acids and of the proteins so that their

<sup>1</sup> Falk, I. S., *J. Biol. Chem.*, 1918, xxxvi, 229.

<sup>2</sup> Loeb, J., *J. Biol. Chem.*, 1918, xxxiii, 531; *J. Gen. Physiol.*, 1918-19, i, 39, 363, 483, 559.

<sup>3</sup> Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 237.

<sup>4</sup> Pfeleiderer, R., *Arch. ges. Physiol.*, 1897, lxvi, 605. A review of the early literature is given in this article.

<sup>5</sup> Berg, W. N., and Gies, W. J., *J. Biol. Chem.*, 1906-07, ii, 489.



solutions probably did not have the same hydrogen ion concentration. Sörensen<sup>6</sup> has shown that the activity of pepsin depends very largely on the hydrogen ion concentration, and at the same time has developed accurate methods for the determination of the latter. In view of his work it is obvious that the comparison of the action of the different acids must be made in such a way as to keep this factor constant. Failure by the earlier workers to do this probably accounts in large measure for the great differences in the observed efficiency of the various acids.

More recently Michaelis and Mendelssohn<sup>7</sup> have shown that the optimum acidity for the digestion of edestin by pepsin is the same for hydrochloric, nitric, tartaric, and oxalic acids. Ringer<sup>8</sup> states that the optimum reaction depends on the protein used and coincides with the maximum hydration of the protein as measured by the viscosity. A somewhat similar theory was proposed by Pfeleiderer<sup>4</sup> who attempted to show a relation between the rate of digestion of fibrin and the amount of swelling in various acids.

In all these investigations the amount of digestion was followed by determining (1) the amount of solution of an insoluble substrate, (2) the amount of precipitable protein left in solution, or (3) the rate of liberation of carmine from carmine fibrin. As has been pointed out by various authors, there is considerable doubt as to whether any of these methods actually follows the chemical changes in the structure of the protein during hydrolysis.

The recent improvements in the technique of the determination of amino nitrogen by the Van Slyke<sup>9</sup> method make it possible to follow the increase in the number of free amino groups. From our knowledge of the changes involved in the hydrolysis of proteins it would seem that this increase probably follows accurately the amount of hydrolysis. The changes are small, however, and even with the greatest care it is difficult to get strictly accurate figures.

<sup>6</sup> Sörensen, S. P. L., *Biochem. Z.*, 1909, xxi, 131.

<sup>7</sup> Michaelis, L., and Mendelssohn, A., *Biochem. Z.*, 1914, lxxv, 1.

<sup>8</sup> Ringer, W. E., *Kolloid-Z.*, 1916, xix, 253; *Arch. néerl. de Physiol.*, abstracted in *Physiol. Abstr.*, 1919, iii, 408.

<sup>9</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1913-14, xvi, 121.

In the present work the rate of pepsin digestion of gelatin, egg albumin, edestin, blood albumin, and casein in the presence of hydrochloric, nitric, acetic, sulfuric, oxalic, phosphoric, and citric acids has been followed by this method. The determinations were made at two ranges of hydrogen ion concentration, pH 1.0 to 1.5 and pH 2.5 to 3.5.

A summary of the results obtained with edestin at a reaction of pH 2.6 is given in Table I. The results obtained with the other pro-

TABLE I.

*Quantity of Amino Nitrogen per 10 cc. of Solution at 24°C. and 750 mm.*

Substrate, 20 cc. of edestin solution A. } Final volume, 120 cc.  
Pepsin, 20 cc. of solution A.

Experiment No.	Time.	Acid.							
		HCl	HNO <sub>3</sub>	Acetic	H <sub>2</sub> SO <sub>4</sub>	Oxalic	H <sub>3</sub> PO <sub>4</sub>	Citric	HCl*
	hrs.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
1	0	0.50	0.49	0.49	0.48	0.51	0.50	0.49	0.49
2	0	0.50	0.50	0.48	0.49	0.48	0.51	0.48	0.49
1	4	1.28	1.23	1.09	1.33	1.22	1.24	1.20	0.50
2	4	1.25	1.21	1.16	1.25	1.26	1.25	1.24	0.47
1	24	1.55	1.58	1.17	1.56	1.48	1.44	1.39	0.51
2	24	1.51	1.50	1.37	1.48	1.50	1.52	1.49	0.50
pH of solution.									
1	24	2.6	2.6	2.6	2.5	2.4	2.2	2.2	2.5
2	24	2.5	2.7	2.6	2.4	2.7	2.4	2.3	2.3

\* Control with boiled pepsin solution.

teins were practically identical with these and therefore will not be given here. The experiments show that the rate of hydrolysis of all the proteins studied is identical for all the acids (except acetic) within the rather wide range of error of the method used (about 5 per cent). With gelatin acetic acid also behaves quantitatively like the other acids even in concentrations as high as 25 volumes per cent. With the other proteins the rate of hydrolysis in the presence of acetic acid is slower than with HCl, HNO<sub>3</sub>, H<sub>2</sub>SO<sub>4</sub>, oxalic, citric, or phosphoric acids. The effect therefore is evidently on the protein

and not on the enzyme. These experiments also show that the physical properties of the solution, such as viscosity, have little or no effect on the rate of digestion since Loeb has shown that there is a striking difference in the viscosity of gelatin in sulfuric as contrasted with hydrochloric acid solution. This is still more strikingly shown in the experiments with edestin, which is practically insoluble in sulfuric acid and yet digests under these conditions at the same rate as when dissolved in hydrochloric acid. The simplest explanation of these results would seem to be that the rate of digestion of the protein is determined by the amount of acid protein salt formed. As Loeb<sup>2,3</sup> has shown, the physical properties of a gelatin solution are also functions of this same quantity.

## II. EXPERIMENTAL.

*Method of Keeping Samples.*—Samples were withdrawn from the bottles containing the digestion mixtures at intervals of 4 and 24 hours and placed in iced bottles containing three drops of saturated ferric chloride solution. They were kept at 2–4°C. until analyzed. No change could be noted in the amino nitrogen content in 24 hours under these conditions.

*Temperature.*—Some of the experiments were conducted at  $37^{\circ} \pm 0.1$ , and some at  $35^{\circ} \pm 0.1$ .

*Analysis.*—The technique was the same as described by Van Slyke except that 10 cc. of solution were analyzed in a large size apparatus and the gas was measured in a small (3 cc.) burette. This was necessary owing to the small amount of amino nitrogen present. The protein solution was allowed to stand 15 minutes in contact with the nitrous acid and then shaken rapidly for 5 minutes. The reaction was complete under these conditions.

*Pepsin Solution A.*—30 gm. of Fairchild's pepsin were dissolved in 500 cc. of water and allowed to digest at  $37^{\circ}$  for 24 hours. The solution was dialyzed and filtered, and made up to 3 liters. It contained 0.1 cc. of amino nitrogen per 10 cc. This quantity did not further increase digestion in 24 hours and therefore does not enter into the figures obtained for the increase of amino nitrogen in the solutions.

It was found that 20 cc. of this solution added to 100 cc. of the protein solution caused the hydrolysis to be about one-third complete in 4 hours and two-thirds complete in 24 hours. This concentration was therefore used.

*Edestin Solution A.*—25 gm. of crystalline edestin were dissolved in 300 cc. of dilute NaOH and precipitated by the addition of dilute HCL. The reaction was adjusted to the isoelectric point of edestin and the solution then dialyzed for a week against tap water and 2 days against distilled water. It was then diluted to 500 cc. A fine suspension was obtained which could be accurately pipetted. The conductivity was about that of an  $M/1,500$  KCL solution, showing that only traces of electrolytes were present. The other protein solutions were purified in the same way by dialysis at the isoelectric point (Loeb).<sup>3</sup>

#### *Adjustment and Measurement of the Reaction.*

The required amount of protein solution was pipetted into a 100 cc. volumetric flask and a drop of indicator added (methyl orange or thymol blue, depending on the reaction desired). Hydrochloric acid was then added until the approximate reaction desired was reached. The solutions containing the other acids were prepared in the same way by adding the acid to the protein solution until the color matched exactly that of the flask containing the hydrochloric acid solution. In this way solutions of the same pH could be easily prepared.

Control experiments showed that the indicator had no effect on either the rate of digestion or the analysis. The absolute reaction of the mixtures could not be measured colorimetrically owing to the "protein error." A sample of the solution was removed shortly after adding the pepsin therefore and the pH determined by the E.M.F. method. It was found as stated by Sørensen that the change in reaction during the digestion was insignificant.



## SUMMARY.

1. At equal hydrogen ion concentration the rate of pepsin digestion of gelatin, egg albumin, blood albumin, casein, and edestin is the same in solutions of hydrochloric, nitric, sulfuric, oxalic, citric, and phosphoric acids. Acetic acid diminishes the rate of digestion of all the proteins except gelatin.

2. There is no evidence of antagonistic salt action in the effect of acids on the pepsin digestion of proteins.

3. The state of aggregation of the protein, *i.e.* whether in solution or not, and the viscosity of the solution have no marked influence on the rate of digestion of the protein.

The author is indebted to Drs. Van Slyke and Cullen for placing their apparatus for the hydrogen ion determination at his disposal.

## THE PHYSIOLOGICAL BASIS OF MORPHOLOGICAL POLARITY IN REGENERATION. II.

By JACQUES LOEB.

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(Received for publication, April 17, 1919.)

### I. INTRODUCTION.

The preceding papers have shown that the phenomenon of regeneration of shoots in *Bryophyllum calycinum* is to be treated as a problem of nutrition and growth, since equal masses of sister leaves, when cut off from the plant, produce under equal conditions and in equal time approximately equal masses of shoots though the number of shoots may differ considerably;<sup>1</sup> and since stems split longitudinally, each retaining one sister leaf, produce also approximately equal masses of shoots in equal times and under equal conditions.<sup>2</sup> When the masses of two sets of sister leaves are made unequal, the mass of shoots produced by the leaf or by the stem varies approximately in proportion to the mass of the leaves. This law holds only when the leaves are exposed to light and it seems difficult to interpret it in any other way except that it is primarily the mass of the material produced in or sent out by the leaf which determines the mass of shoots produced in these cases.

Phenomena of regeneration show a second characteristic which we may designate briefly as that of morphological polarity, whereby we mean that an isolated piece of a plant or animal will as a rule produce different organs at opposite ends. If regeneration is a phenomenon of nutrition or chemical mass action, it will also become necessary to account for this polarity in terms of nutrition or mass action. We have commenced an investigation of the physiological basis of polarity and have published already some results which have

<sup>1</sup> Loeb, J., *Bol. Gaz.*, 1918, lxv, 150.

<sup>2</sup> Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 81.

also shown a dominating influence of the mass of the leaf.<sup>3</sup> Thus in *Bryophyllum calycinum* a leaf at the apex of a piece of stem will inhibit the formation of shoots in the basal part of the stem and this inhibitory influence disappears when the mass of the leaf is diminished below a certain limit. This inhibitory influence of the leaf upon shoot formation follows the path of the conducting vessels from the leaf into the stem. Hence a leaf favors shoot formation at the apex of a piece of stem and inhibits shoot formation at the basal parts of the stem, and both phenomena are a function of the mass of the leaf.

This influence of the leaf exists in the normal plant as well as in the regenerating plant; *i.e.*, in a piece cut out from the plant. The normal stem of *Bryophyllum calycinum* has two dormant buds in each node capable of growing into shoots, though they never do so in a normal plant. This is partly due to the influence of the apical leaves and this influence is responsible for the fact that the normal stem of *Bryophyllum* is unbranched. We say partly due to the leaves since the growing region at the apex acts in a way similar to a leaf.<sup>3</sup>

We intend to show in this paper that the leaf not only influences shoot formation but also root formation.

## *II. Influence of the Mass of an Apical Leaf on the Mass of Air Roots Formed.*

Pieces containing a number of nodes were cut out from healthy plants of *Bryophyllum calycinum*. All the leaves except the two apical ones were removed and the stems were split longitudinally so that each half of stem possessed one apical leaf. One leaf was usually left intact; the mass of the other leaf was reduced by cutting away part of the leaf. The two sets of half stems were suspended horizontally in a moist aquarium with the cut surface above (Figs. 1 and 2). On the lower side of the stems roots soon formed, first generally at the second node behind the leaf, and later at the base and at other nodes. At the base the formation of roots was most abundant. Still later roots were also formed in the internodes, especially in the region of geotropic curvature.

<sup>3</sup> Loeb, J., *Science*, 1917, xlv, 547; *J. Gen. Physiol.*, 1918-19, i, 337.



FIG. 1. Stems split longitudinally and suspended horizontally, each half stem having one leaf at the apex; stem on left, whole leaf; stem on right, leaf reduced in size. Considerably more roots are produced in the half stems with whole leaf on left than on right where the mass of leaf is diminished. Geotropic curvature is also greater where the mass of leaf is greater. Duration of experiment 36 days.



FIG. 2. Repetition of experiment in Fig. 1. Duration of experiment 25 days.



It was expected and found that the larger the mass of apical sister leaves the greater the mass of roots produced in equal time and under equal conditions. Figs. 1 and 2 are typical experiments of this kind. It was not possible, however, to prove that the two masses of roots formed (in about 5 weeks) are always proportional to the masses of leaves. This may be due to two sources of error inherent in experiments on root formation. First, the roots we are dealing with are air roots which usually dry out and fall off after a short time, while new air roots begin to form. This alone would make it almost impossible to prove an exact proportionality between mass of leaf and of root production even if it existed. The second difficulty lies in the fact of the small mass of each root, which makes the error in cutting off and in ascertaining the mass of the roots rather large. The fresh as well as the dry weight of the roots was determined in each case. The data given in Table I, however, leave no doubt that the mass of roots increases when the mass of leaves increases, though no mathematical expression of the relation can be deduced, for the reasons stated. When the half stem has no leaf the root formation is greatly retarded and diminished if not suppressed, as is shown in Fig. 3. One set of half stems had an apical leaf, while the other set had no leaf. The stems were lying with their cut surface on moist filter paper. The stems without a leaf formed practically no roots though they formed apical shoots. The other set of half stems with an apical leaf formed an abundant supply of roots but the shoot formation was suppressed by the apical leaf. The drawing was made 26 days after the beginning of the experiment.

The writer has repeatedly called attention to the fact that not only the root formation but also the geotropic curvature of the stems increases with the mass of the apical leaves,<sup>4</sup> and this fact was confirmed in the new experiments. The stems in Figs. 1 and 2 were originally perfectly straight, and the curvature noticeable in the photographs is due to the growth in length of a certain layer of cells of the cortex. These experiments show, in the opinion of the writer, that the phenomenon of geotropic curvature is also a phenomenon of

<sup>4</sup>Loeb, J., *Bot. Gaz.*, 1917, lxiii, 25; *Ann. Inst. Pasteur*, 1918, xxxii, 1.

TABLE I.

*Showing that the Mass of Air Roots in a Stem Diminishes with the Mass of the Apical Leaf. The Stems were Split Longitudinally, Each Half Possessing One Sister Leaf at the Apex. In One Set the Leaf Was Left Intact, in the Other It Was Reduced in Size by Cutting Off Part of the Leaf. The Stems with Reduced Leaf Invariably Formed a Small Mass of Roots.*

No. of experiment.	Date and duration of experiment.		Weight of leaves.	Weight of air roots on stem.	Approximate ratio of weight	
					Of leaves.	Of roots.
1	Jan. 15 to Feb. 21, 1918.	6 whole leaves, <i>fresh</i> .....	19.03	0.054	6.7	6.7
		6 sister leaves reduced in size, <i>fresh</i> .....	2.85	0.008		
2	Oct. 29 to Dec. 9, 1918.	5 whole leaves, { <i>fresh</i> .....	51.1	0.077	3	3
		{ <i>dry</i> .....	2.627	0.016		
		5 sister leaves reduced in size, { <i>fresh</i> .....	16.33	0.025		
		{ <i>dry</i> .....	0.901	0.005		
3	Jan. 9 to Feb. 17, 1919.	5 whole leaves, { <i>fresh</i> .....	11.65	0.043	4	2
		{ <i>dry</i> .....	0.526	0.006		
		5 sister leaves reduced in size, { <i>fresh</i> .....	2.935	0.022		
		{ <i>dry</i> .....	0.132	0.003		
4	Jan. 22 to Mar. 6, 1919.	6 whole leaves, { <i>fresh</i> .....	34.462	0.128	3.3	6.1
		{ <i>dry</i> .....	1.378	0.037		
		6 sister leaves reduced in size, { <i>fresh</i> .....	10.032	0.026		
		{ <i>dry</i> .....	0.416	0.006		
5	Jan. 31 to Mar. 3, 1919.	5 whole leaves, { <i>fresh</i> .....	32.8	0.044	3	2
		{ <i>dry</i> .....	1.504	0.009		
		5 sister leaves reduced in size, { <i>fresh</i> .....	8.625	0.026		
		{ <i>dry</i> .....	0.485	0.005		
6	Oct. 27 to Dec. 9, 1918.	5 whole leaves, { <i>fresh</i> .....	14.4	0.423	11.6	6.4
		{ <i>dry</i> .....	1.36	0.109		
		5 sister leaves reduced in size, { <i>fresh</i> .....	1.57	0.900		
		{ <i>dry</i> .....	0.117	0.017		

nutrition and growth and that Haberlandt's hypothesis of an otolith function of starch granules as the cause of geotropic curvature is neither needed nor warranted. The hypothesis of geotropic "stimulation" had better be replaced by the application of the law of mass action.<sup>5</sup>

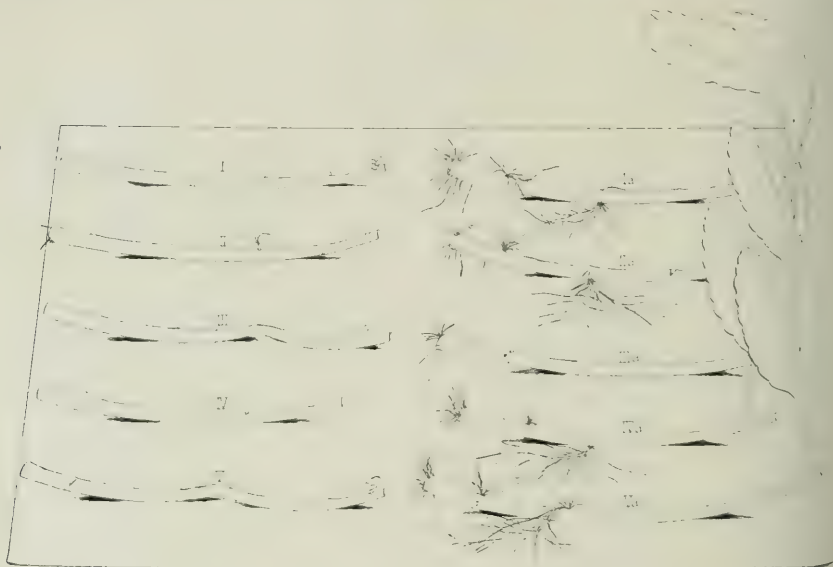


FIG. 3. I and I a, II and II a, etc., halves of the same stem split longitudinally and put with their cut side on moist filter paper. Half stems I a, II a, etc., on right, having each a leaf at apex, form considerable masses of roots on stem, while half stems I, II, etc., on left, having no apical leaf, form practically no roots in the same time. Duration of experiment 26 days.

### *III. The Influence of Light Upon Root Formation in the Stem.*

Six stems were split longitudinally into two pieces, each with an apical leaf as described. One-half of each stem was suspended horizontally, with the cut surface above, in a dark aquarium, the other in an aquarium exposed to light. The temperature was the same in both aquaria. After 22 days photographs (Figs. 4 and 5) were taken. The stems exposed to the light (Fig. 4) had formed a

<sup>5</sup> Loeb, J., *Forced movements, tropisms, and animal conduct*. Monographs on Experimental Biology, Philadelphia, 1918, 120.

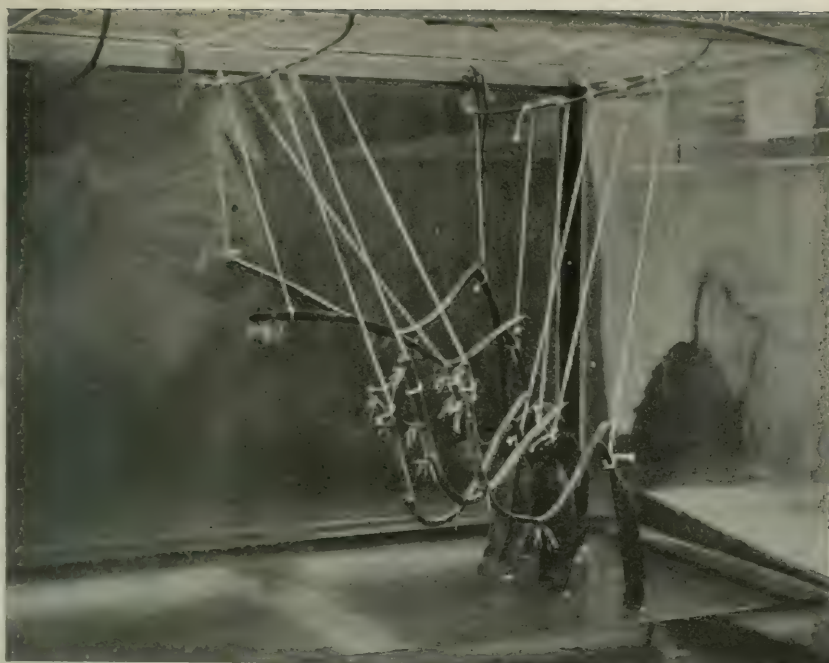


FIG. 4.

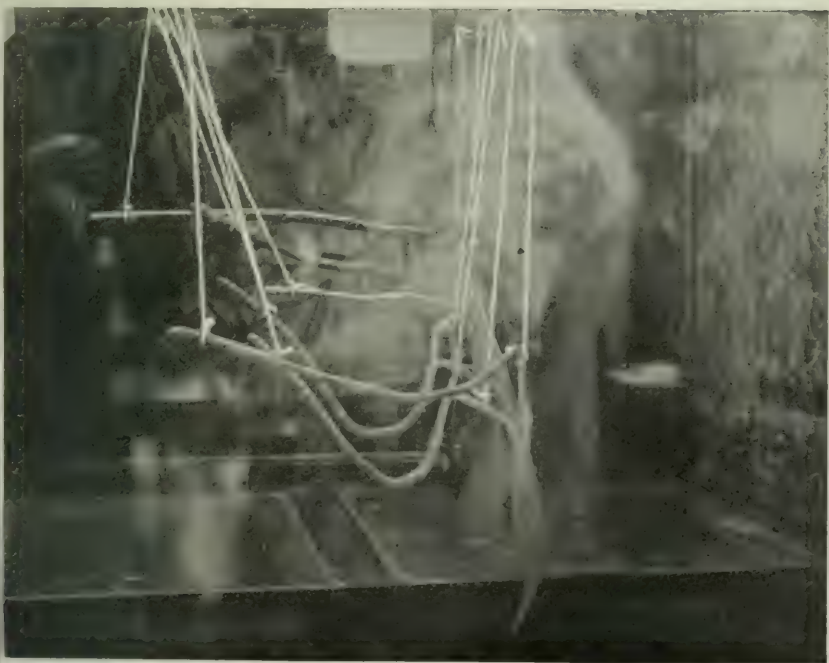


FIG. 5.

FIGS. 4 and 5. Six stems split longitudinally each half stem possessing one typical leaf. Half stems in Fig. 5, kept in dark, produce practically no roots while the half stems in Fig. 4, exposed to light, form an abundance of air roots. Duration of experiment 22 days.



considerable mass of air roots while the stems kept in the dark (Fig. 5) had formed practically no roots. In the dark the leaves have a tendency to fall off but we are not ready to attribute the non-appearance of roots on the stems kept in the dark purely to the interruption of the sap flow from leaf to stem. It is probably also partly due to the interruption of the process of assimilation in the leaf. This is suggested by the fact that we get the same inhibition or



FIG. 6. Stems split longitudinally, each half stem having one apical leaf. Stems on left, having their leaves covered with tin-foil, produce only few roots, while stems on right, having their leaves exposed to light, produce in the same time a considerable mass of air roots. Duration of experiment 27 days.

suppression of root formation when we cover the leaf with tin foil in which case the tendency of the leaf to fall off is less striking. Figs. 6 and 7 are illustrations of such an experiment after 27 days. There was less difference in the geotropic curvature of stems with leaves in the light and in the dark than in the mass of roots produced. This may be due to the fact that the geotropic curvature occurs at an

earlier date than the root formation; hence the substances existing in the leaf at the time of darkening may suffice for bringing about the geotropic curvature but will not suffice for bringing about ample root formation.

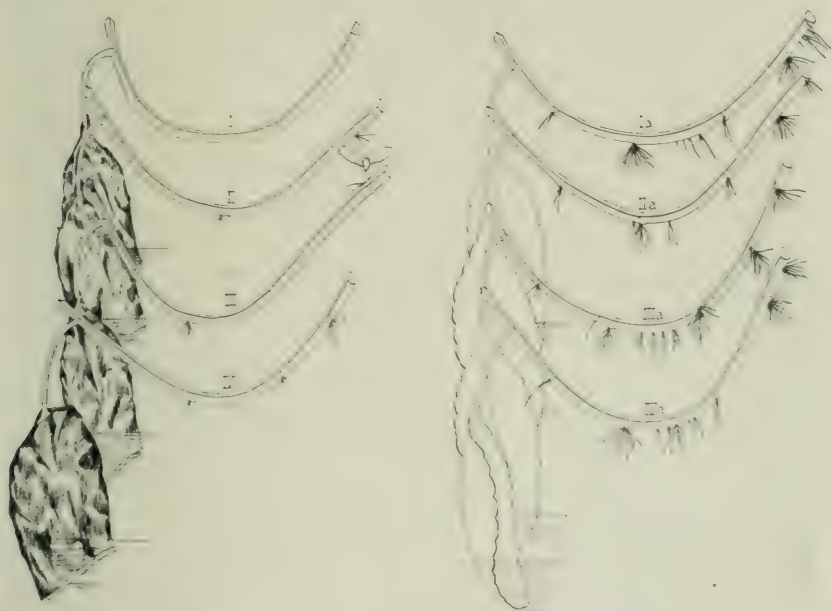


FIG. 7. Drawing of the same stems as in Fig. 6.

#### *IV. Effect of Gravity on Root Formation.*

Figs. 8 and 9 are photographs of whole stems suspended horizontally, each stem having an apical leaf on the upper side. All these stems were originally perfectly straight. The reader will notice that all the stems have an abundant supply of roots but these roots are all on the lower side of the stem with the exception of the extreme basal end. The mass of the leaves was reduced in one set of stems (Fig. 8) and the mass of roots was less in this set than in the other (Fig. 9), where the mass of the leaves was larger, thus confirming our statement at the beginning of this paper. The stems with a larger mass of leaves have undergone also considerably greater curvature than the stems with a smaller mass of leaves.

There is another connection between root formation and geotropic curvature. We have shown in a preceding paper<sup>4</sup> that the curvature is due to the fact that the under side of the cortex of a stem suspended

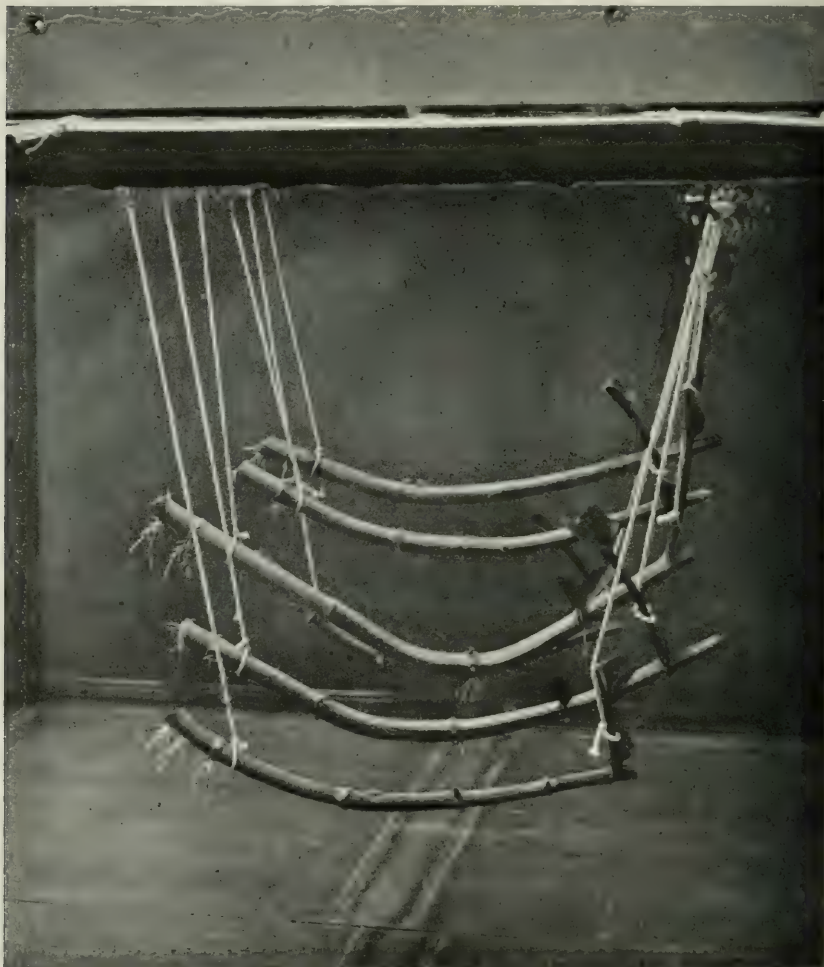


FIG. 8.

horizontally continues to grow in length while the upper side does not do so to any appreciable degree. The photographs (Figs. 8 and 9) show that if we disregard the basal end of the stem, the roots develop

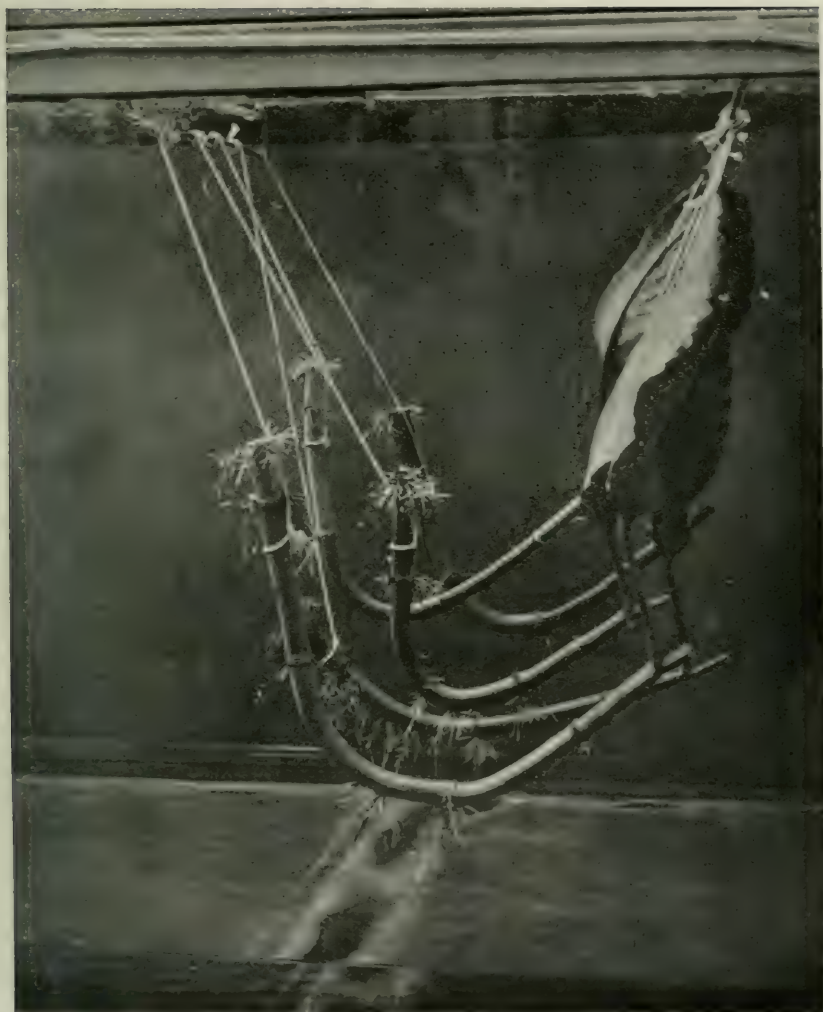


FIG. 9.

FIGS. 8 and 9. Whole stems with leaf at apex on upper side. Stems in Fig. 8 with leaves reduced in mass produce fewer roots than stems in Fig. 9 with whole leaves. In both cases roots are formed on the under but not on the upper side of stems, except at the extreme cut end. The geotropic curvature is greater in stems with whole leaves (Fig. 9) than in stems with reduced leaf (Fig. 8). Duration of experiment 36 days.



only on the basal side of the stem. The fact that no roots grow on the upper side of the stem cannot, however, be dismissed with the mere statement of an influence of gravity in this case. Fig. 4 shows that an abundance of roots will be formed on the upper side of a horizontal stem when the lower side is cut off. Why then will no roots develop on the upper side of an intact stem suspended hori-

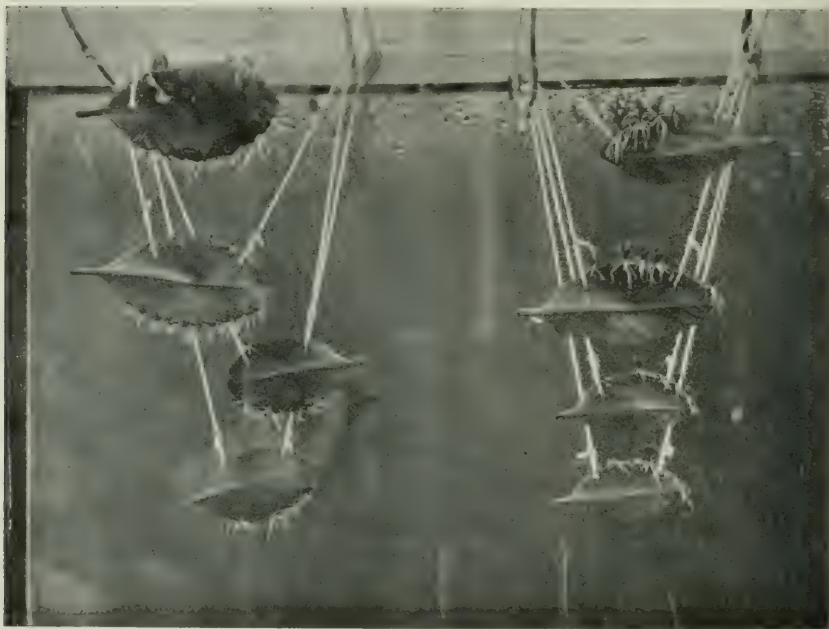


FIG. 10. Leaves suspended sidewise in air. On left side of photograph more roots are formed on the lower than on the upper side of leaf; in the leaves on the right side of the photograph the lower edge is cut off. In this case the upper edge of the leaves produces the roots and shoots which otherwise would have developed below. Duration of experiment 25 days.

izontally? The reason is that the roots grow more rapidly on the lower side of a stem suspended horizontally and that on account of this more rapid growth of the roots on the under side the growth of roots on the upper side of the stem is inhibited. When we remove the under side of a stem this inhibitory effect disappears and roots will grow from the upper side of the stem.

The correctness of this reasoning (which is of importance for the theory of polarity) can be demonstrated by experiments on the production of roots in isolated leaves. When isolated leaves of *Bryophyllum calycinum* are suspended sidewise in a moist atmosphere, an abundance of roots is formed on the lower side of these leaves and none or fewer on the upper side (see left half of Fig. 10). This is due to the fact that roots commence to develop a little earlier in the notches on the under than on the upper side of such a leaf and that they grow in general a little more rapidly on the lower than on the upper side. The more rapidly growing elements in a leaf inhibit the growth of the more slowly growing elements of the same kind.<sup>1</sup> The correctness of this interpretation is demonstrated on the right half of the photograph. The four leaves on the right side of Fig. 10 are the sister leaves of the four leaves on the left. The lower edge of the leaves on the right side was cut off, thus preventing the growth of roots on the lower side, since roots only grow out from preformed buds in the notches of a leaf. In this case the roots on the upper side grow out more abundantly. The photograph was taken on the 25th day of the experiment. Figs. 11 and 12 demonstrate another experiment of the same type. Fig. 11 shows that in the intact leaves on the left half of the drawing, the roots grow out sooner on the lower than on the upper side. The drawing was made after 8 days. In the sister leaves on the right side of the drawing, the lower edge of each leaf was cut off, thus preventing root formation on the lower side of the leaves. Practically no roots had formed on the upper side of the leaves. Fig. 12 is a drawing of the same leaves 25 days later. In the leaves on the right side, where the notches on the under side were cut off, the growth of roots and shoots on the upper side is much more abundant than it is on the upper side of the sister leaves on the left side of Fig. 12, where the growth on the upper side was retarded or inhibited by the more liberal growth on the under side of the leaves. The mass of shoots in each pair of sister leaves was practically in proportion to the mass of the leaves.

A striking demonstration of the principle that acceleration of root (and shoot) formation in one part of a leaf inhibits the root (and shoot) formation in the other part of the leaf is offered in Figs. 13 and 14. The four leaves in Fig. 13 were put with their apices into water which

accelerates root formation in the notches. An enormous growth of roots occurred in the watered notches. This suppressed the growth of roots in the other notches of the leaf and caused the air roots already formed there to shrink and dry up soon after they were formed.

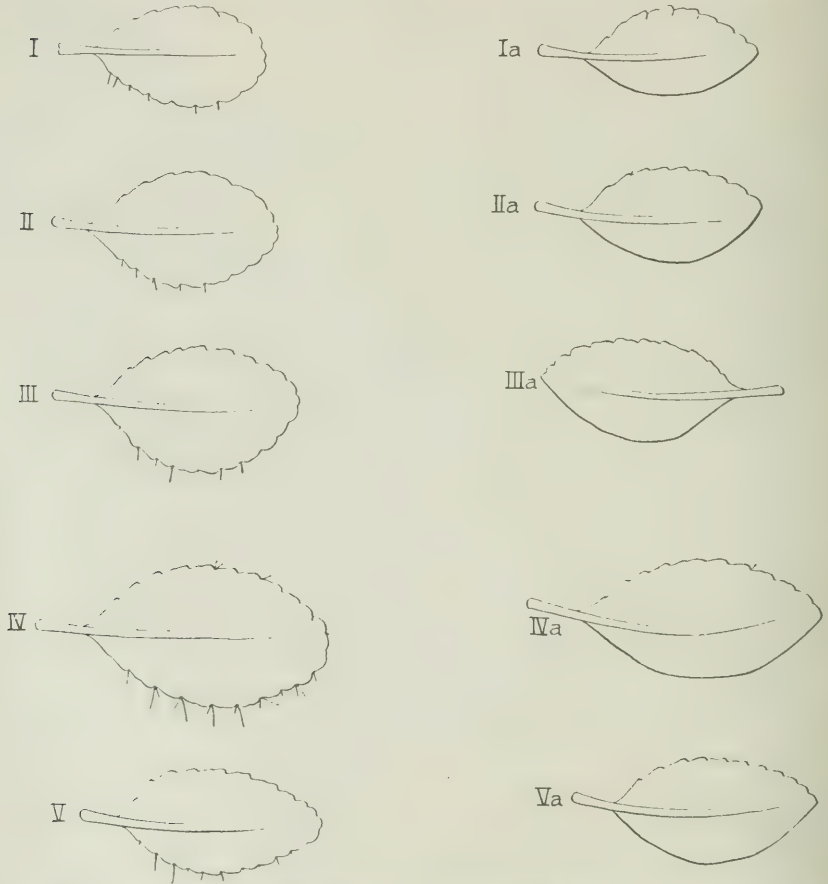


FIG. 11. Similar experiment as in Fig. 10, showing that roots form first at lower edge of leaves suspended sidewise. Drawn on 8th day of experiment.

The drawing was made on the 17th day. Fig. 14 is a drawing of the sister leaves, made also after 17 days. These sister leaves were not dipped in water, but were suspended in moist air. Hence no special acceleration of root formation took place in any part of the leaf.

As a consequence we have an abundant supply of roots in practically every notch of the leaf. In the upper lateral leaves which are suspended sidewise the growth of roots is again more abundant in

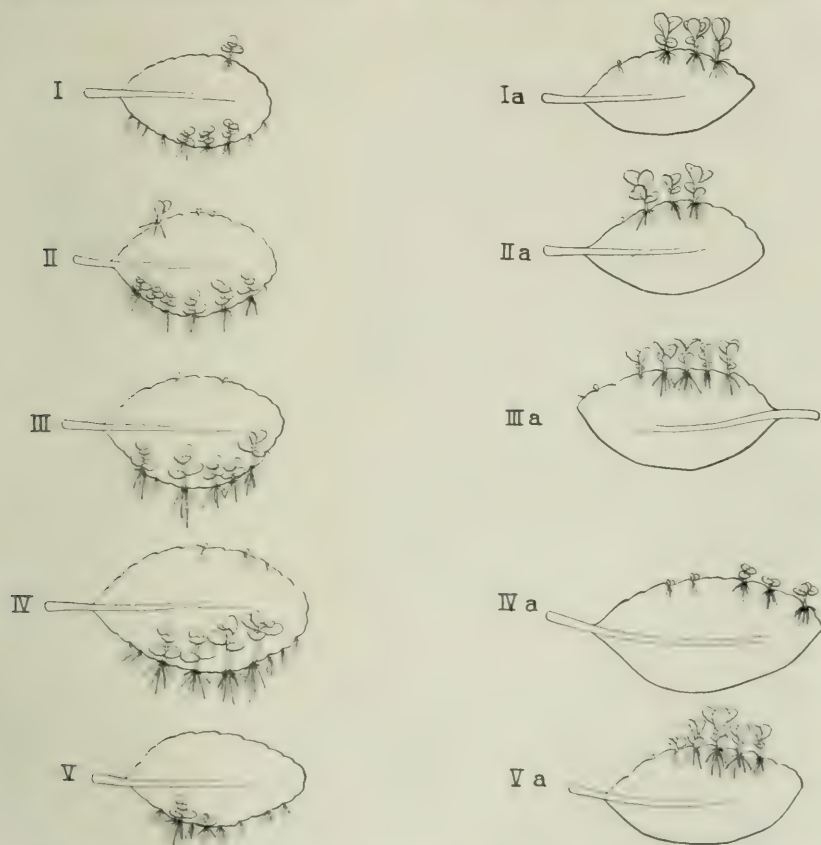


FIG. 12. The same leaves as in Fig. 11, but 25 days later, showing that the more rapid development of roots on the lower side of Leaves I to V retards or inhibits the development of roots on the upper side of the leaves. In Leaves Ia to Va, where the lower edge is cut off, the development of roots and shoots on the upper side is not restricted.

the notches on the lower side, while no such influence of gravity is noticeable in the lower central leaves. This difference has been discussed in a previous paper and the influence of gravity on the



development of roots in the leaves suspended sidewise was explained as being due to a greater collection either of water or of solutes or of both in the notches on the lower side.<sup>1</sup> This influence of gravity on

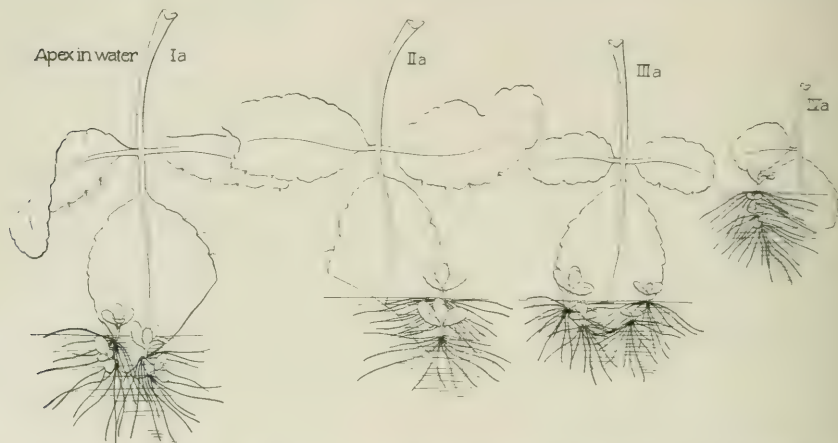


FIG. 13. Four leaves dipped with their apices in water whereby the development of roots is accelerated in the apex. As a consequence the growth of roots in the upper notches of the leaves is inhibited. Duration of experiment 17 days.

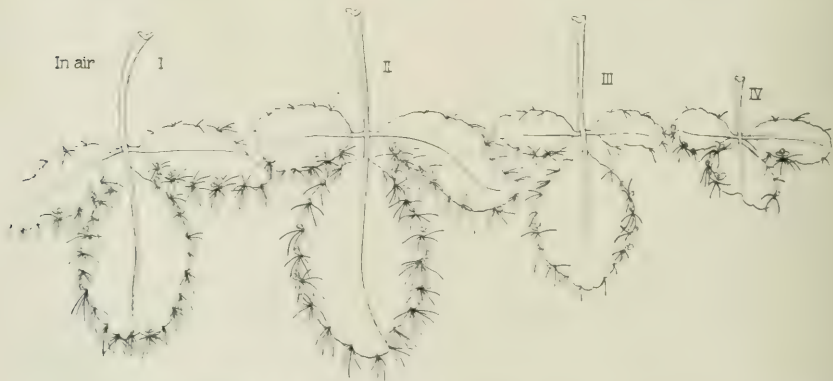


FIG. 14. Sister leaves of those in Fig. 13 suspended in moist air. Most of the notches have roots since the growth is nowhere sufficiently accelerated to retard it in the rest of the notches. Duration of experiment 17 days.

the collection of water can show mainly in the more fleshy parts of the leaf, *i.e.* in the middle, but not in the thin tissues of the apex of the leaf.

When we dip isolated leaves with their apices into water an enormous root and shoot formation will occur in the notches of the apex but nowhere else. The proof that the rapid growth of roots and shoots in the apex inhibits the formation of roots and shoots in the other notches was furnished by the following experiment. After some time the leaves were taken out of the water and suspended in moist air and the shoots formed in water were removed. The roots formed in water dried out. Very soon roots and shoots began to appear in the upper notches of the leaf thus proving that the growth in the upper notches had originally been suppressed by the more rapid growth of the roots and shoots formed at the apex when the latter was dipping into water.

If we turn from these experiments to the observations on root formation in whole stems suspended horizontally (Figs. 8 and 9), we reach the conclusion that the upper side of such a stem (with the exception of the extreme basal end) is free from roots for the reason that water or solutes or both collect a little more freely on the lower side of the stem, thus favoring root formation on that side. The greater rapidity of growth of the roots on the lower side creates secondary conditions by which the growth of roots on the upper side is inhibited. When we remove the lower half of a horizontally suspended stem we remove this inhibitory influence and now roots can grow out on the upper side.

Since in all these cases the horizontally suspended stems bent geotropically, whereby the under side became convex, it might be argued that this convexity was the cause of the greater abundance of roots on the under side. A series of simple experiments showed that this was not the case. Pieces of stem were bent passively and tied in this position to sticks of wood (Fig. 15). They were suspended in such a way that they turned their concave side downwards. In all cases, without any exception, the roots developed on the lower sides of the stems which were now concave (Fig. 15, after 35 days). They also developed on the under side when the bending of the stem was prevented by cork rings and when the stem was enclosed in a glass tube, as in Fig. 16.

Reed and Halma<sup>6</sup> have shown that shoots grow out only on the upper side of horizontally suspended branches of citron trees. Our experiments published in a previous article show that shoots can grow out from the under side of a piece of stem of *Bryophyllum*, suspended horizontally, and that they invariably do so when the upper half

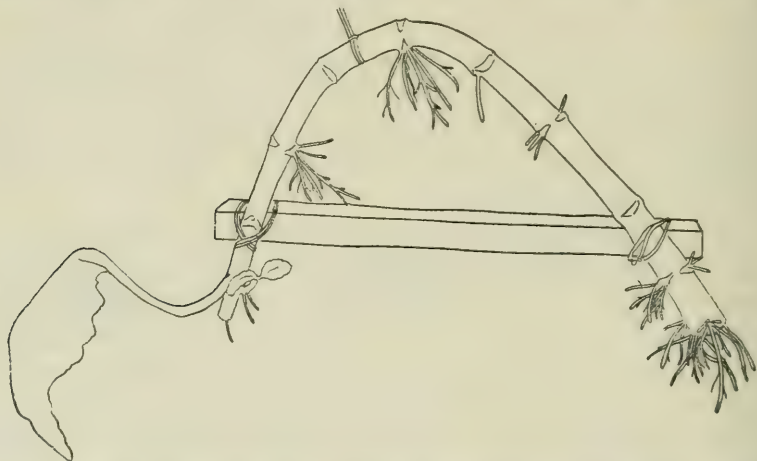


FIG. 15. Stem bent passively, concave side below. Roots form on lower concave side. Duration of experiment 35 days.

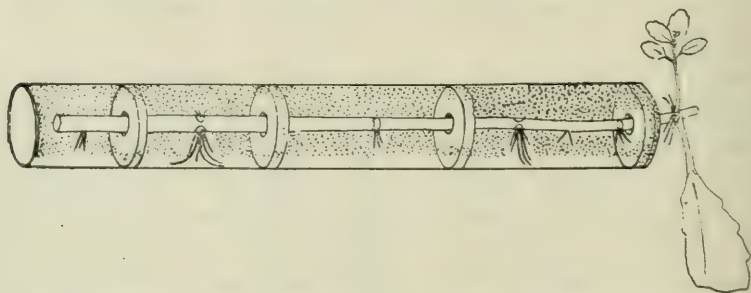


FIG. 16. Geotropic curvature prevented in stem. Roots are formed nevertheless on lower side. Duration of experiment 41 days.

of such a stem is removed. This suggests the possibility that if the upper half of a citron bough, suspended horizontally, is removed the shoots might grow out from the under side, and that in Reed and

<sup>6</sup> Reed, H. S., and Halma, F. F., *Univ. Cal. Pub., Agric. Sc.*, 1919, iv, 99.

Halma's experiment the formation of shoots on the under side of a piece of stem of citron when suspended horizontally was inhibited by the more rapid growth of the shoots on the upper side of such a piece of stem.

The writer has already reported an experiment<sup>3</sup> which proves directly that the growth of a shoot on the upper side of a stem of *Bryophyllum*, suspended horizontally, inhibits the growth of a shoot on the lower side of such a stem. When we isolate a piece of stem of *Bryophyllum* and remove all the leaves from such a piece the two buds situated in the most apical node will grow out into shoots. When we place a stem horizontally in such a way that the line connecting the two apical shoot buds is vertical, both buds will begin to grow out but the upper shoot will grow more rapidly than the lower one and in the majority of cases the lower shoot will soon stop growing, while the upper shoot continues to grow. When we remove the upper bud, this inhibitory effect on the lower bud disappears and the shoot on the lower side will grow out as vigorously as the upper shoot would have done if its bud had not been removed.

Our experiments show also that the path for the substances favoring root formation is not identical with the path determining inhibition of shoot formation. Thus in Figs. 8 and 9 the inhibitory influences on shoot formation in the stem follow the conducting vessels on the upper half of the horizontally suspended stem, while the root formation is favored in the lower half of the stem.

#### *V. The Preformation of Root Buds in the Axil of a Leaf.*

We have seen that in the axil of each leaf of *Bryophyllum calycinum* there is one bud capable of growing out into a shoot, though this growth is inhibited in a healthy plant under normal conditions, and the mechanism of this inhibition determines also the polar character of regeneration as far as shoot formation is concerned. Roots can develop anywhere on the stem and this causes the appearance as if no preformation of root buds existed in the plant. Yet this appearance is deceptive since as a matter of fact in the axil of each leaf there is not only a preformed shoot bud but also a preformed root bud. Curiously enough, this root bud lies a little above (apically from) the shoot bud. Special experiments are required to demonstrate the



existence and activity of these root buds. The simplest method of doing this is represented in Fig. 17. Vigorous stems consisting of five or six nodes are deprived of all leaves except those at the base. These basal leaves should be large since, as we have seen, the mass of root formation increases *ceteris paribus* with the mass of the leaves. The stems are suspended vertically in moist air so that the basal cut end just dips into water. In about 3 or 4 days (at greenhouse temperature and with good illumination) shoots and roots begin to grow out simultaneously. Fig. 17 gives a picture of such an

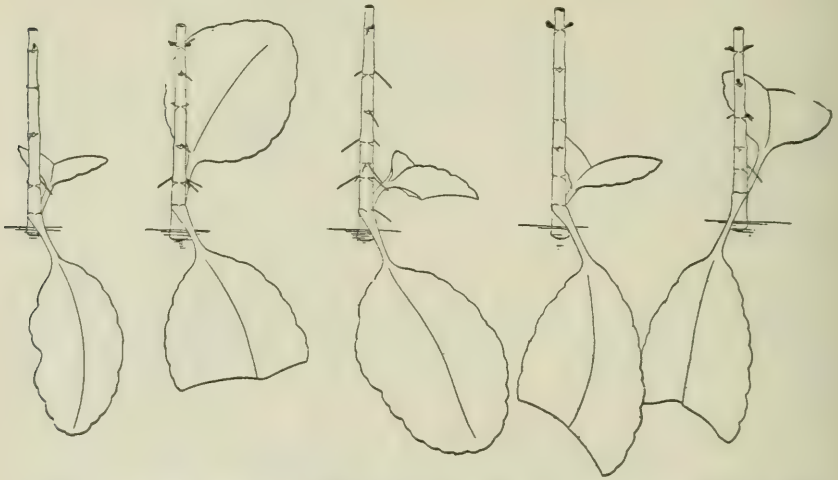


FIG. 17. Stems with two large leaves at base, the latter dipping in water, showing that roots form in many of the nodes situated apically from the leaf from buds situated above the shoot bud. Drawn after 6 days.

experiment after 6 days. The shoots are represented in thick black spots and the roots in double lines, thus imitating their natural appearance since the buds are at first dark red and the roots are, of course, white. It is obvious that each root takes its origin above (apically from) the bud.

About a week or 8 days after the preformed root buds have grown out from the stem, the formation of roots at the base near the cut end begins (Fig. 18). Under the conditions of our experiment they touched the water and began to grow very rapidly. As soon as

this happened, the air roots in the upper part of the stem began to dry out and were not replaced by new roots. The inference must be drawn that the luxurious growth of the roots at the basal end of

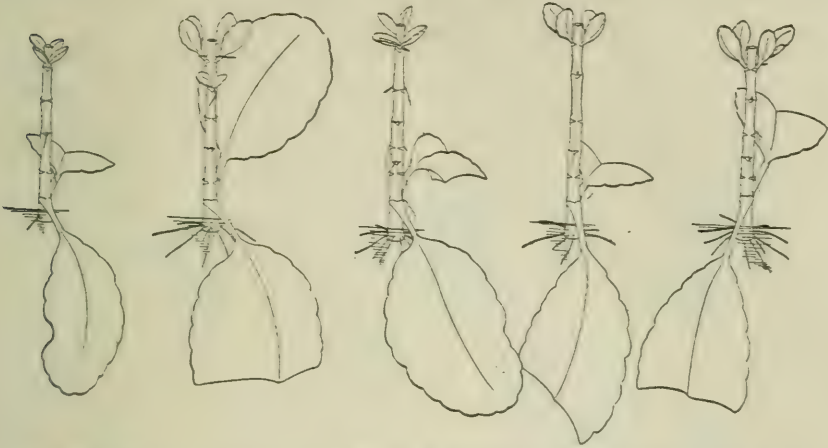


FIG. 18. The same stems as in Fig. 17, 8 days later. The basal roots in water have now commenced to develop and as a consequence the air roots, located higher up and formed before, commence to dry out and fall off.



FIG. 19. The same stems as in Fig. 18 but 9 days later. The air roots have practically disappeared. The basal roots in water are developing rapidly.

the stem inhibited the further growth of the roots in the more apical nodes of the stem; in the same way as the more rapid growth of the most apical buds of a stem suppresses in general the growth of buds situated more basally. Thus in each stem (Fig. 17) not only the

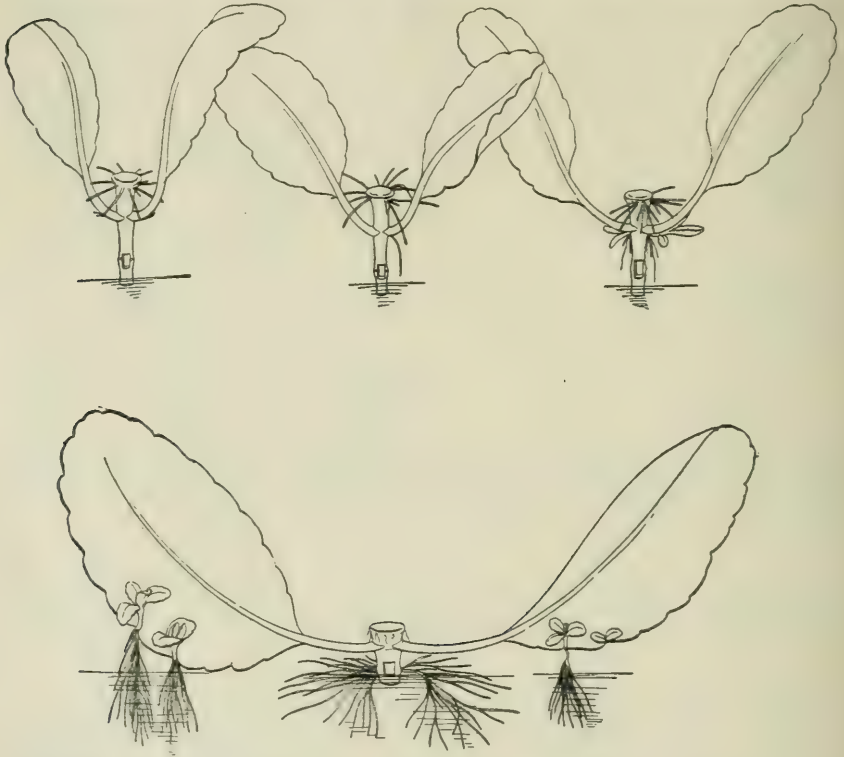


FIG 20. Short stems with large basal leaves dipping with their apical ends in water. All the stems produced air roots at the basal end, which in this case is above and in air. The lower one of the four stems formed roots at its apical end and these roots dipping in water grew more rapidly than the air roots. In this case the air roots at the upper (basal) end of the stem dried out. Drawn after 26 days.

most apical buds but also the buds in one or two nodes below began to grow out, but their growth was suppressed by the more rapid growth of the most apical bud (Fig. 18). Fig. 19 is a drawing of the same stems 9 days later. Hardly any trace of the air roots in the upper

part of the stem is now left, while the basal roots have developed. Only at the base of the apical shoots roots may continue to grow.

The suppression of the growth of the more apical roots at the time when the roots at the base of the stem begin to grow is the analogue of the suppression of roots in a leaf the apex of which dips into water, as in Figs. 13 and 14.

These ideas raised the question whether it would be possible to suppress the roots at the base of a stem by putting the apical roots into water, thereby accelerating their growth. Fig. 20 shows such an experiment. Short pieces of stems with a pair of leaves at their base and one node in front were dipped with their apices into water. The root and shoot buds of the apical nodes were removed and only one stem (the lower one in the drawing) formed roots at the apex which grew rapidly. The air roots at the base of this stem which had begun to form dried out and never grew again while the air roots at the base of the three other stems (which had formed no roots at the apex) continued to grow. The drawing was made on the 26th day of the experiment. It is, however, not very easy to cause abundant root formation at the apex and hence this experiment will have to be repeated.

#### *VI. The Rôle of Nodes in Root Formation.*

The reader will have noticed that in order to demonstrate that roots can be formed also in the apical regions of a piece of stem we used isolated pieces of a stem with two leaves at the basal end. The basal leaves must be large since the mass of new roots formed increases with the mass of the leaf. If the leaves are not at the base but in the middle of the leaf, or, more correctly, if the piece of stem basally from the leaf includes one or more nodes, the root formation in the stem apically from the leaf is generally suppressed. The nodes basally from the leaf act like basal roots in preventing root formation in the more apical region. This has been verified in different ways some of which may be described.

When we select long stems with one large leaf at the base of the stem, and suspend them horizontally in moist air, roots develop first at some of the nodes apically from the leaf, and later in abundance at the base of the stem near the cut end (Fig. 21). Though these



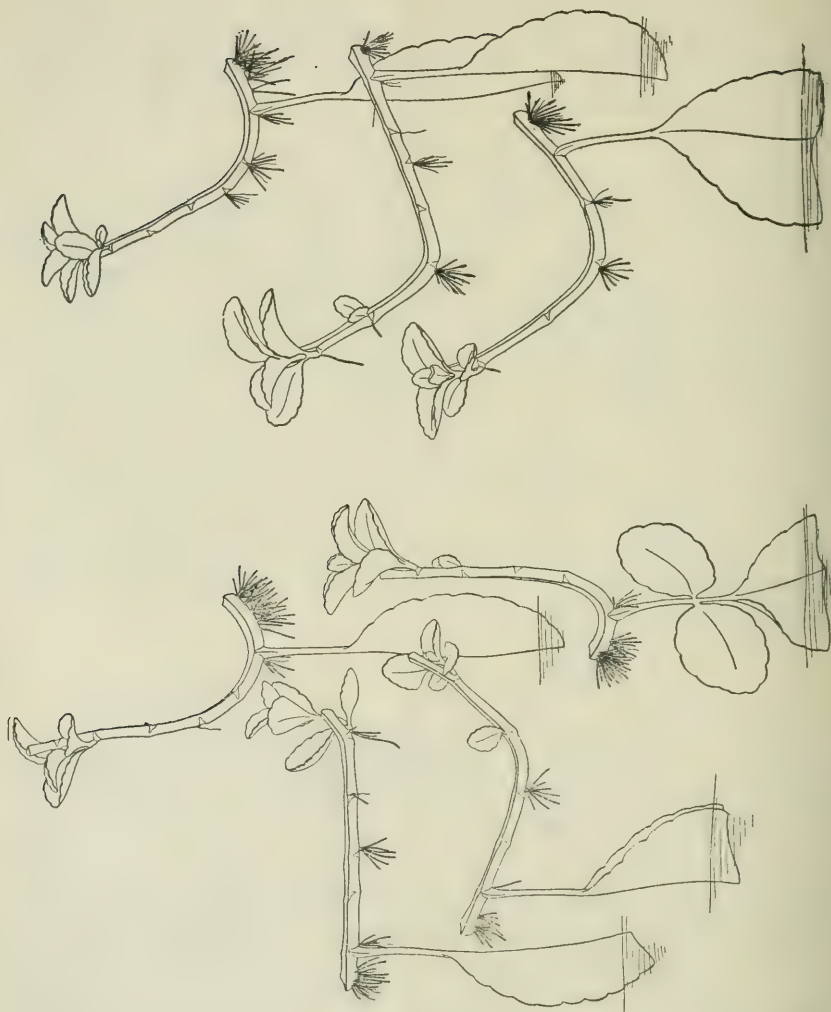


FIG. 21. Stems with leaf at base suspended in moist air. Roots developed first in some of the more apical nodes of the stem and persisted even after the basal roots had formed. The latter, being air roots, grew too slowly to inhibit the growth of the more apical roots. Drawn after 30 days.

latter roots will soon surpass in mass the apical roots, the apical roots will not wilt. The basal roots, when they develop in air instead of in water, do not grow rapidly enough to suppress the growth of the more apically situated roots. The drawing was made on the 30th day after the beginning of the experiment. The upper half of the stems had been cut off in this experiment. If, however, the leaf is not at the base but in the middle of the stem, as in Fig. 22, roots will

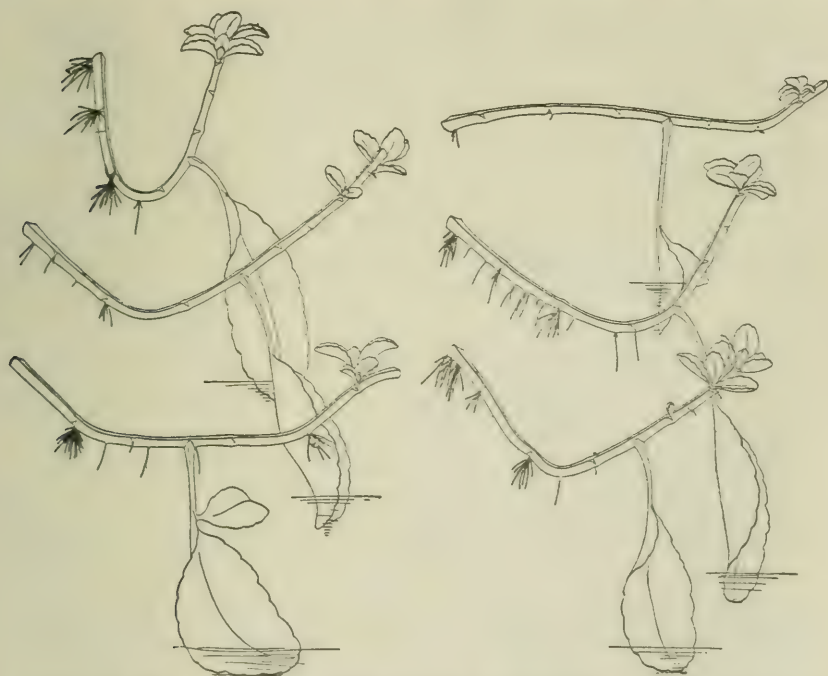


FIG. 22. Leaf in middle of stems suspended horizontally. Practically no roots develop in front of the leaf, the nodes basally from the leaf inhibiting the growth in the more apical nodes. Drawn after 24 days.

develop and persist only basally from the leaf. It is only necessary for this result that one node is preserved basally from the leaf, as in Fig. 23.

When we suspend a stem of *Bryophyllum calycinum*, possessing one leaf in the middle and on the under side of the stem, horizontally in moist air, the root formation will usually start first from the second

node and not from the first node behind the leaf.<sup>7</sup> The reason for this lies probably in the fact that the dormant root bud in the second node lies in the path of the descending sap from the leaf, while the root bud in the first node basally from the leaf lies above this path. We notice generally that in a piece of stem suspended horizontally,

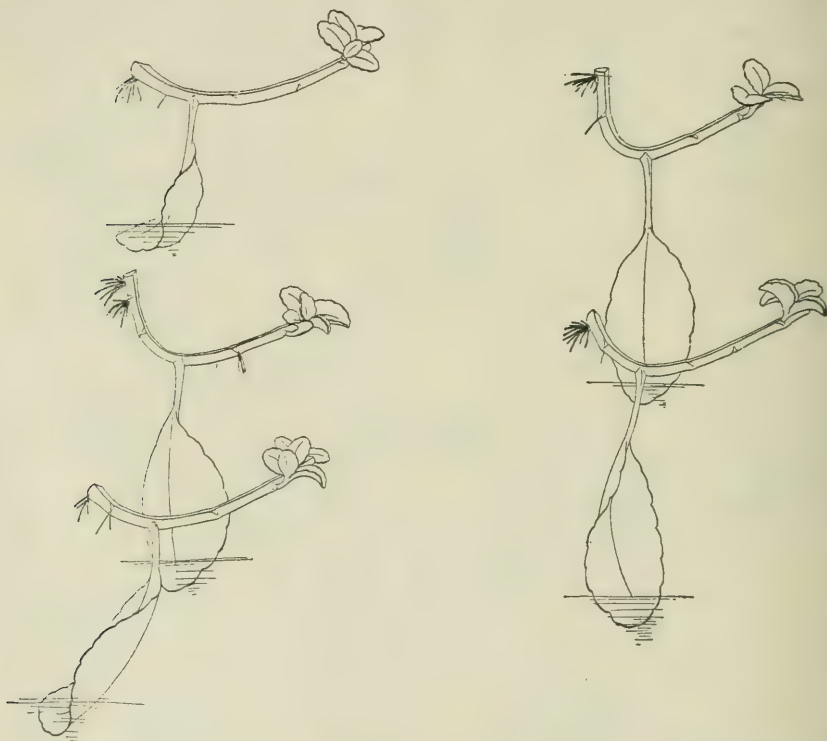


FIG. 23. Showing that one node behind the leaf suffices for this inhibition of the growth of roots in the buds situated apically from the leaf.

possessing one apical leaf on the under side and two nodes basally from the leaf, roots will grow out before they grow out in a similar piece of stem possessing only one node basally from the leaf.

<sup>7</sup> Loeb, J., *Bot. Gaz.*, 1917, lxiii, 25.

*VII. General Remarks.*

As far as the polar character of root formation is concerned, the experiments show that dormant buds for the development of air roots exist in the axil of each leaf (apically from the resting shoot buds) and that it is the rapid growth of the basal roots in the soil or in water which prevents these roots from growing out in the normal plant.

To explain this inhibition it suffices to assume that a more rapidly growing organ in a plant generally inhibits the more slowly growing organ of the same kind in other parts of the plant. This principle is, perhaps, the most generally active in the phenomena of correlation. The more abundant growth of the basal roots must find its explanation on the assumption of the greater collection of water and solutes necessary for root growth at the base of a normal or isolated piece of stem. This follows from the fact proved in this paper that the mass of air roots formed in an isolated piece of stem increases with the mass of leaf attached to the stem and that the leaf has this influence only if it is exposed to light. This indicates that the root formation is primarily a phenomenon of nutrition and growth.

Bayliss<sup>6</sup> has called attention to a paper by Errera<sup>9</sup> on "inhibitory stimulation" in which this author discussed the influence of the apex of fir trees on the direction of growth in secondary branches, and of the apex of roots on the direction of growth of secondary roots. Errera accepted Czapek's hypothesis of the formation of a specific antioxi-dase as a consequence of the "geotropic perception" and raised the question whether the influence of the apex on the lower ramifications does not consist essentially in sending to these branches, or in calling forth in these branches the formation of, a substance antagonistic to this antioxi-dase—a kind of antibody. This idea is, of course, very hypothetical, and the writer without being aware of Errera's suggestion had offered a different explanation of the same phenomenon.<sup>7</sup>

The assumption of the existence of specific inhibitory substances which the writer had used tentatively in two preceding papers,<sup>3</sup> and

<sup>6</sup> Bayliss, W. M., *Nature*, 1918, cii, 285.

<sup>9</sup> Errera, L., *Bull. Soc. Roy. bot. Belgique*, 1904, xlii, 27.



which was based on the experiments of geneticists, is not needed for the explanation of the results published in this paper.

In the next paper the writer intends to furnish direct proof for the fact that the isolation of a piece of a plant leads to a change in the flow and distribution of sap and that this change in the distribution determines the starting of growth of formerly dormant buds which is the essential feature in regeneration.

#### SUMMARY

1. The experiments show that the mass of air roots formed in a stem increases with the mass of the leaf attached to the stem, though it has not been possible to establish an exact mathematical relation between the two masses, owing to unavoidable sources of error.

2. Darkened leaves do not increase the mass of roots formed.

3. In stems suspended horizontally air roots appear on the lower side of the stem, with the exception of the cut end where they usually appear around the whole circumference of the stem. When the lower half of a stem suspended horizontally is cut off, roots are formed on the upper side. It is shown by experiments on leaves suspended horizontally that the more rapidly growing roots and shoots on the lower side inhibit the root and shoot formation in the upper half of such a leaf; and likewise the more rapid formation of roots on the lower side of a horizontally suspended stem seems to account for the inhibition of root formation on the upper side of such a stem. Likewise the more rapid growth of shoots on the upper side of a stem suspended horizontally is likely to inhibit the growth of shoots on the lower side.

4. Each leaf contains in its axil a preformed bud capable of giving rise to a root, which never grows out in the normal stem on account of the inhibitory influence of the normal roots at the base of the plant. These dormant root buds are situated above (apically from) the dormant shoot bud. The apical root buds can be caused to develop into air roots when a piece of stem is cut out from a plant from which the leaves except those in the basal node of the piece are removed. The larger these basal leaves the better the experiments succeed.

5. These apical air roots grow out in a few days, while the roots at the basal end of the stem (which in our experiments dip into water) grow out about a week later. As soon as the basal roots grow out in water they cause the air roots in the more apical region of the stem to dry out and to disappear.

6. In addition to the basal roots, basal nodes have also an inhibitory effect on the growth of the dormant root buds in the apical region of a stem. This is indicated by the fact that a stem with one pair of leaves near the base will form apical air roots more readily when no node is situated on the stem basally from the leaf than if there is a node basally from the leaf.



## THE INFLUENCE OF ELECTROLYTES ON THE ELECTRIFICATION AND THE RATE OF DIFFUSION OF WATER THROUGH COLLODION MEMBRANES.

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### I. INTRODUCTION.

The direct measurements of the osmotic pressure of sugar solutions by Morse<sup>1</sup> and his fellow workers as well as by Lord Berkeley and Hartley<sup>2</sup> show so close an approximation to the values calculated on the basis of van't Hoff's theory that it is difficult to doubt the correctness of this theory. As Fick<sup>3</sup> pointed out as early as 1866, we can, on the assumption of the validity of the kinetic theory, treat a solution as a mixture of two gases, the solvent and the solute. When the solution is separated from the pure solvent by a semipermeable membrane more molecules of the solvent will impinge in the unit of time on the unit area of the membrane on the side of the pure solvent than on the side of the solution and hence the solvent will diffuse to the solution until the partial pressure of the solvent on the solution side becomes so great, that the number of molecules of solvent impinging on the unit of membrane on the solution side becomes equal to the number of molecules impinging on the unit of membrane on the side of the pure solvent.

There are, however, phenomena which suggest that in addition to the gas pressure other forces may enter into the problem of diffusion of water through a membrane. Such phenomena are the cases of

<sup>1</sup> Morse, H. N., The osmotic pressure of aqueous solutions, *Carnegie Institution of Washington, Publication 198*, 1914.

<sup>2</sup> Earl of Berkeley, and Hartley, E. G. J., *Proc. Roy. Soc. London, Series A*, 1916, xcii, 477.

<sup>3</sup> Fick, A., *Die medizinische Physik*, Braunschweig, 1866, 2nd edition, 36; *Z. physik. Chem.*, 1890, v, 526.



so called negative osmosis in which the solvent moves from a solution of higher to one of lower molecular concentration and which have been described and studied by Dutrochet, Thomas Graham, Girard, Flusin, Bartell, Bernstein, Freundlich,<sup>4</sup> and others.

Girard, Bernstein, and Bartell have reached the conclusion that potential differences caused by the electrolyte play a part in these phenomena of abnormal osmosis, and our results are in agreement with this view. We cannot agree, however, with the view of Girard and of Freundlich that the H and OH ions play the decisive part in the electrical phenomena which lead to abnormal osmosis. This view of a privileged position of the H and OH ions in these phenomena rests on the famous experiments of Perrin<sup>5</sup> on electric endosmose. It had been known that when a galvanic current is sent through a porous diaphragm bounded on both sides by water the latter migrates to one of the two electrodes. No reason could be assigned for the direction of the migration, until Perrin made the remarkable discovery that when a current is sent through a colloidal diaphragm (gelatin, silk, etc.) the direction of motion of the water can be altered at will; addition of acid causes the water to move to the anode, addition of alkali to the cathode. This phenomenon was explained by Perrin on the assumption that the H or OH ions of the water are adsorbed by the surface of the diaphragm transferring their charges to the latter. A double layer is formed, the molecules of water adjacent to the diaphragm assuming the opposite electrical charge. However, Perrin noticed and pointed out a serious difficulty in this assumption, namely that no other monovalent ions except the H and OH ions are capable of such an apparent transfer of their charges to the diaphragm.

The writer is under the impression that we must discriminate between two kinds of diaphragms, namely those which consist of material

<sup>4</sup> Bernstein, J., *Elektrobiologie*, 1912. Bartell, F. E., *J. Am. Chem. Soc.*, 1914, xxxvi, 646. Bartell, F. E., and Hocker, C. D., *J. Am. Chem. Soc.*, 1916, xxxviii, 1029, 1036. Freundlich, H., *Kolloid-Z.*, 1916, xviii, 11. Girard, P., *Compt. rend. Acad.*, 1908, cxlvi, 927; 1909, cxlviii, 1047, 1186; 1910, cl, 1446; 1911, cliii, 401; La pression osmotique et le mécanisme de l'osmose, Publications de la Société de Chimie-physique, Paris, 1912. Flusin, G., *Ann. chim. et phys.*, 1908, xiii, 480.

<sup>5</sup> Perrin, J., Notice sur les titres et travaux scientifiques de M. Jean Perrin, Paris, 1918.

which is an amphoteric electrolyte (e.g. gelatin or silk) and those which consist of non-amphoteric material. Both types are not necessarily influenced in the same way by acids and alkalies. Amphoteric electrolytes form salts with acids as well as with alkalies and this salt formation reverses the influence of the diaphragm on the sign of electric endosmose. HCl transforms gelatin into gelatin chloride, and NaOH transforms it into sodium gelatinate. In the case of gelatin chloride the gelatin ion is positive and in the case of sodium gelatinate it is negative. We assume that it is primarily the sign of the gelatin ion which determines the sense of the electrification of water and that the OH and H ions play only a secondary part. This would explain why Perrin could find no other monovalent ion except H or OH which was able to reverse the electrification of the gelatin diaphragm, since only acids and alkalies can transform a gelatin cation into a gelatin anion, or *vice versa*, while neutral salts cannot produce such an effect.<sup>6</sup>

We are, therefore, of the opinion that the experiments on electrical endosmose through diaphragms of colloidal substances like gelatin, pig's bladder, silk, or any other amphoteric electrolyte, do not warrant the assumption of a specific influence or predominant position of the H and OH ions in the electrification of a membrane bounded on both sides by water, except in so far as acids and alkalies are the only substances which can reverse the sense of ionization of an amphoteric electrolyte.

Girard, in accepting Perrin's idea of a direct electrification of the wall by H and OH ions, and of the specific action of these two ions, tried to ascribe the phenomena of abnormal osmosis he observed with membranes of pig's bladder to the acid or alkaline reaction of the solution. Thus neutral solutions, like those of NaCl and Na<sub>2</sub>SO<sub>4</sub>, should be without any other except a purely osmotic effect and he publishes data apparently supporting this conclusion.

The writer's results with collodion membranes do not agree with the conclusions of Girard, and we shall see that the solutions of neutral salts act as powerfully on the rate of diffusion of water through collodion membranes as the solutions of alkalies, acids, or acid salts. We

<sup>6</sup> Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 39, 237.

shall show that there is no difference between the osmotic effects of the hydroxides and of the chlorides or nitrates, *e.g.*  $m/128$  NaCl and  $m/128$  NaOH acting qualitatively and quantitatively practically alike in their influence on the rate of diffusion of water through collodion membranes.

The writer has carried out a series of experiments on the rate of diffusion of water through a collodion membrane separating the pure solvent from a solution. These experiments indicate that this process depends on two kinds of forces; namely, first, those of gas pressure which are clearly recognizable in the case of solutions of non-conductors like glucose or saccharose, and second, electrical forces which become predominant in the case of low concentrations of solutions of electrolytes. We shall deal in this paper with the electrical forces. They lead to the result that in the diffusion of water through a collodion membrane to a solution of an electrolyte the water molecules are either positively or negatively charged according to the nature of the electrolyte in the solution and that these charges are a factor in the rate of diffusion. The laws or rules controlling this factor can be expressed in the following way.

1. Solutions of neutral salts possessing a univalent or bivalent cation influence the rate of diffusion of water through a collodion membrane, as if the water particles were charged positively and were attracted by the anion and repelled by the cation of the electrolyte; the attractive and repulsive action increasing with the number of charges of the ion and diminishing inversely with a quantity which we will designate arbitrarily as the "radius" of the ion. The same rule applies to solutions of alkalies.

2. Solutions of neutral or acid salts possessing a trivalent or tetravalent cation influence the rate of diffusion of water through a collodion membrane as if the particles of water were charged negatively and were attracted by the cation and repelled by the anion of the electrolyte. Solutions of acids obey the same rule, the high electrostatic effect of the hydrogen ion being probably due to its small "ionic radius."

We shall show first that the assumption of a difference in the sign of the charge of water molecules (according to the two rules just mentioned) explains all the phenomena that can be observed; and



we shall show later that the correctness of the assumption concerning the sign of the charge of the molecules of water can be proved directly by experiments on electric endosmose.

## *II. Observations on the Rate of Filtration.*

Collodion bags in the form of Erlenmeyer flasks of a content of about 50 cc. were prepared in as uniform a way as possible and were used after several days testing. Fresh bags were usually too permeable to serve for our purpose. The mouth of the collodion bag was closed with a perforated rubber stopper and the mouth of the bag was fitted tightly to the stopper with the aid of rubber bands. Through the hole in the stopper a glass tube with a bore of about 2 mm. in diameter was pushed into the bag. The collodion bag was filled with distilled water which reached into the glass tube to a height of about 110 mm. above the level of the distilled water of a beaker into which the bag was dipped. This pressure head of about 110 mm. of water inside the bag increases the number of particles impinging during the unit of time on the unit of area of the inside of the collodion bag and as a consequence more water diffuses in the unit of time from the bag into the beaker than diffuses in the opposite direction. Consequently water diffuses out and the pressure head on the water in the bag diminishes constantly. By measuring the level of the water in the glass tubes at definite intervals and plotting the values of the level as ordinates over the time as abscissæ we get a picture of the rate of diffusion of water out of the bag (Fig. 1).

When the outside solution is not distilled water but a solution of a non-conductor, *e.g.* cane sugar, the rate of diffusion of water is accelerated owing to the fact that the presence of sugar particles diminishes the number of water particles which in the unit of time impinge on the unit of area on the outside of the collodion membrane. Hence if the inside of the bag contains water and if the pressure head of the water inside the bag is at the beginning again about 110 mm. of water, the difference in the number of particles of water impinging on the inside remains the same as before while the number of water molecules impinging on the outside is diminished through the presence of the sugar particles. This diminution must be in proportion to the concentration of the sugar solution.



Fig. 1 gives the curves for the rate of fall in the pressure head of water in the glass tube when the beaker contains distilled water or different molecular concentrations of cane sugar. The curves show that the accelerating influence of a  $M/64$  solution of cane sugar on the rate of outflow of water is still very slight under the conditions of these experiments but that the influence of the sugar solution increases with a further increase in its concentration. These curves may serve as standards of comparison for the curves obtained with

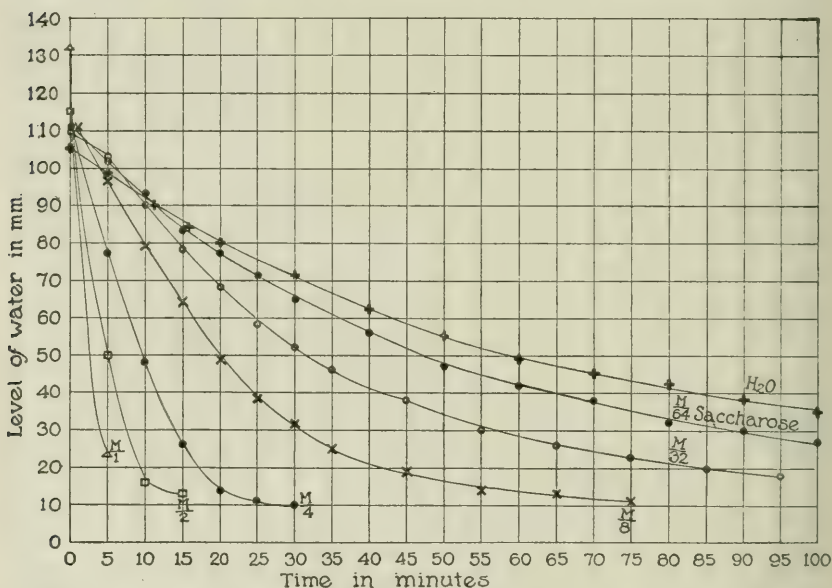


FIG. 1. Curves of fall of level of water diffusing against distilled water and cane sugar solutions of various concentrations.

electrolytes. The influence of solutions of lower concentration of cane sugar than  $M/64$  is not noticeable by this method.

When we replace the sugar solutions in the beaker with solutions of electrolytes of approximately the same osmotic pressure we notice considerable variations in the rate of outflow of water (or rather the fall of the pressure head in the glass tube) with the nature of the electrolyte, and these variations find their expression—as far as the writer's present observations go—in the two rules mentioned above.

Although in these experiments water not only diffuses from the collodion bag into the beaker but electrolyte diffuses also from the beaker into the collodion bag, we shall show in Chapter IV that the differences in our curves are not due to differences in the rate of the diffusion of the electrolytes but to differences in the rate of diffusion of water from the collodion bag into the beaker. Equilibrium will be reached when the solutions inside and outside of the beaker are identical, but our experiments deal only with the initial rate of diffusion of water leading to this condition of equilibrium and not with the condition of equilibrium itself.

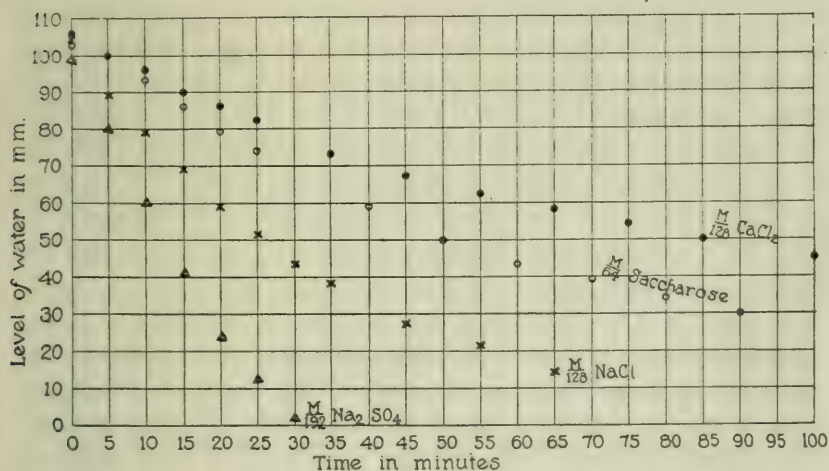


FIG. 2. Curves of fall of level of water when diffusing against solutions of  $M/64$  cane sugar,  $M/128$   $\text{CaCl}_2$ ,  $M/128$   $\text{NaCl}$ , and  $M/192$   $\text{Na}_2\text{SO}_4$ , showing the opposite effect of increase in valency of anion and cation on the rate of diffusion of water.

Fig. 2 illustrates the influence of solutions of three neutral salts,  $\text{NaCl}$ ,  $\text{CaCl}_2$ , and  $\text{Na}_2\text{SO}_4$ , on the rate of diffusion of distilled water from the bag into the beaker containing the salt solution. The water in the bag had an initial pressure head of about 110 mm. of water. Water diffuses more rapidly against a  $M/128$  solution of  $\text{NaCl}$  and still more rapidly against a  $M/192$   $\text{Na}_2\text{SO}_4$  solution than against a  $M/64$  solution of cane sugar. This difference cannot be ascribed to a difference in osmotic pressure of the three solutions since their

osmotic pressure was approximately the same and we shall see later that the phenomenon in question is widely independent of differences in the osmotic pressure of electrolytes. On the other hand, water diffuses more slowly against a  $M/128$  solution of  $CaCl_2$  than against a  $M/64$  sugar solution.

These facts may serve as an illustration of our statement that in the case of electrolytes with univalent and bivalent cation water behaves as if it were positively charged and attracted by the anion and repelled by the cation of the salt, and that both attraction and repulsion increase with the number of charges of an ion. In the case of  $NaCl$  the attractive action of  $Cl$  is for some reason greater than the repelling action of  $Na$ , hence the water diffuses more rapidly towards  $M/128$   $NaCl$  than against  $M/64$  cane sugar. It diffuses still more rapidly against  $M/192$   $Na_2SO_4$  than against  $M/128$   $NaCl$  because the  $SO_4$  ion has two charges united in one ion. In the case of  $CaCl_2$  the two positive charges united in the one  $Ca$  ion suffice to annihilate practically completely the attractive action of the two negative  $Cl$  ions. As a consequence  $M/128$  solutions of  $CaCl_2$  act almost like distilled water on the rate of diffusion of water. Solutions of  $MgCl_2$ ,  $SrCl_2$ ,  $BaCl_2$ ,  $CoCl_2$ , and  $MnCl_2$  act similarly to solutions of  $CaCl_2$ . Fig. 3 shows that the rate of diffusion of distilled water under a pressure head of about 110 mm. towards  $M/192$   $MgCl_2$  is practically identical with the rate of diffusion of water under the same pressure head against distilled water and that a  $M/4$  solution of  $MgCl_2$  is required to accelerate the diffusion of water through the collodion membrane to approximately the same amount as is done by a  $M/128$  solution of  $NaCl$ .

Fig. 4 shows that the attractive action of anions of salts with univalent cations ( $Na$  and  $K$ ) increases with the valency of the anion. Very dilute solutions of oxalates, phosphates, citrates, and the tetravalent anion  $Fe(CN)_6$  attract water so violently that it diffuses extremely rapidly through the membrane. Hence an increase in the number of charges of a cation lowers the attraction of the electrolyte for water, while an increase in the number of charges in an anion increases the attraction. All this agrees with the assumption that the molecules of water possess a positive charge.

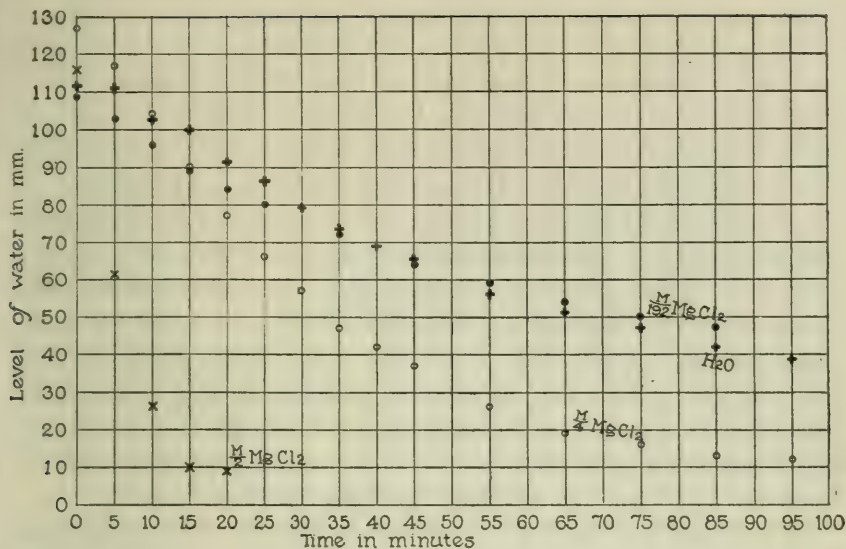


FIG. 3. Curves of fall of level of water when diffusing against solutions of  $\text{MgCl}_2$  of different concentrations.

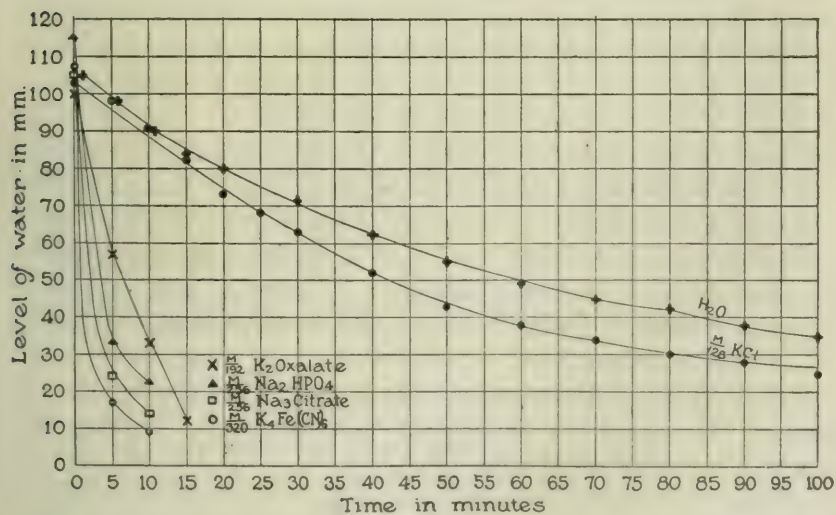


FIG. 4. Curves of fall of level of water when diffusing against solutions of different salts of sodium and potassium, showing the increase in rate with increase in valency of anion.



But this is only true as long as we deal with neutral salts with univalent and bivalent cation. When we deal with neutral salts with trivalent or tetravalent cations the rule is suddenly reversed. Chlorides or nitrates of salts with trivalent or tetravalent cations attract water considerably more than the salts with bivalent or even with monovalent cation. This discontinuity of effect with increase of the number of charges of the cation led the writer to the conclusion that water behaves towards salts with trivalent and tetravalent cations as if its molecules were negatively charged. On account of the high charge of the trivalent and tetravalent cations the attraction for water should be very powerful, as it indeed is. Of course, many of these solutions are strongly acid on account of the hydrolytic dissociation, but this is true only to a slight degree for  $\text{Ce}_2\text{Cl}_6$  or  $\text{La}_2\text{Cl}_6$ . Yet a  $\text{M}/512$  solution of these two salts attracts water almost as powerfully as does  $\text{M}/192$   $\text{Na}_2\text{SO}_4$  of the same hydrogen ion concentration.

That water is attracted by salts with trivalent and tetravalent cations as if its molecules were negatively charged becomes obvious from a comparison of the effects of the chlorides and sulfates of these salts. Since the anion of a salt should repel negatively charged water—and the more so the greater the valency of the anion—the attractive power of a trivalent cation for water should be less in the case of a sulfate than of a chloride. Hence  $\text{M}/512$   $\text{Cr}_2\text{Cl}_6$  should have a greater attraction for water than  $\text{M}/320$   $\text{Cr}_2(\text{SO}_4)_3$ . This is indeed the case. It was found that water in the bag diffused under a pressure head of about 110 mm. of water more rapidly towards  $\text{M}/512$   $\text{Al}_2\text{Cl}_6$  or  $\text{Cr}_2\text{Cl}_6$  than towards  $\text{M}/320$   $\text{Al}_2(\text{SO}_4)_3$  or  $\text{Cr}_2(\text{SO}_4)_3$ . In a  $\text{M}/2048$  solution of  $\text{Al}_2\text{Cl}_6$  it took only 20 minutes for the water in the manometer to fall from a pressure head of 105 to that of 10 mm.; while a  $\text{M}/32$  to  $\text{M}/16$  solution of  $\text{Al}_2(\text{SO}_4)_3$  was required to bring about the same rate of filtration.

It required about 40 minutes for the water to fall from the pressure head of 105 mm. to that of 20 mm. when the outside solution consisted of a  $\text{M}/2048$  solution of  $\text{Cr}_2\text{Cl}_6$ . When  $\text{Cr}_2(\text{SO}_4)_3$  was used in the beaker, even a  $\text{M}/16$  solution of the sulfate did not act as strongly as a  $\text{M}/2048$  solution of the chloride, although the latter was at least partly in suspension. The nitrates of these salts behaved like the chlorides. It is, therefore, obvious that in the case of trivalent cations

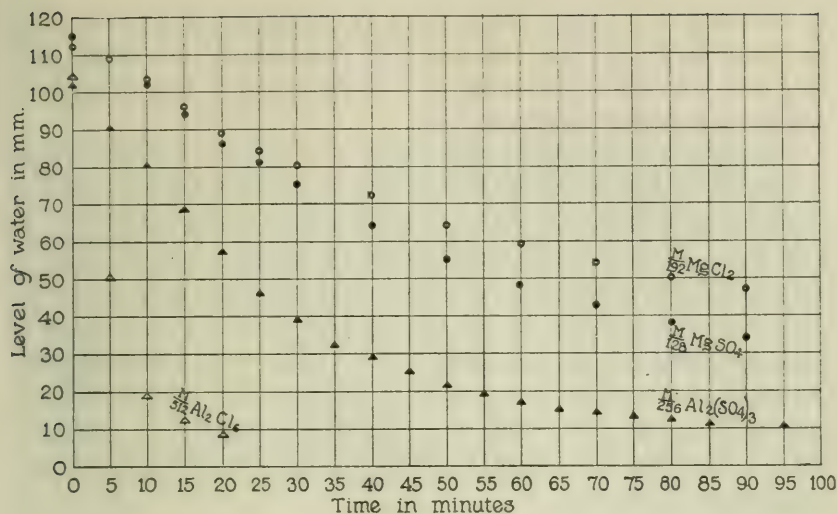
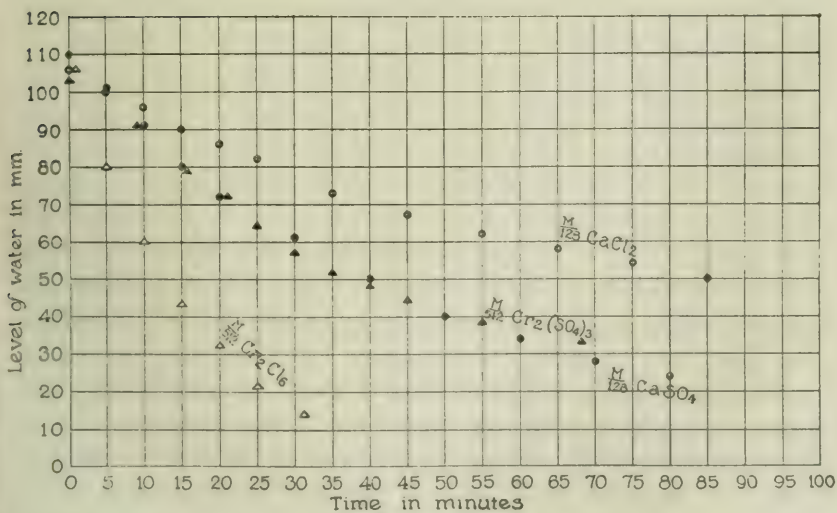


FIG. 5.



FIGS. 5 and 6. Curves showing that in comparison with Cl, SO<sub>4</sub> increases the rate of outflow in the case of bivalent cation and diminishes it in the case of trivalent anion.

the presence of  $\text{SO}_4$  ions retards the rate of diffusion of water in comparison with  $\text{Cl}$  or  $\text{NO}_3$  ions and this supports our conclusion that  $\text{H}_2\text{O}$  acts towards the solution of salts of trivalent cations as if its molecules were negatively charged.

The reverse is true when the cation of a salt is bivalent. When the outside solution was  $\text{M}/128$   $\text{MgSO}_4$  or  $\text{M}/128$   $\text{CaSO}_4$  it required about 75 minutes for the pressure head of  $\text{H}_2\text{O}$  inside the bag to fall from 120 mm. to about 30 mm. When the sulfates were replaced by the chlorides it required much higher concentrations to produce the same rate of diffusion. In the case of  $\text{MgCl}_2$  and  $\text{CaCl}_2$  it required a concentration of approximately  $\text{M}/8$ . This is to be expected when the molecules of water are positively charged since in this case the attractive power of  $\text{SO}_4$  should be superior to that of the chlorides. These statements are supported by Figs. 5 and 6 illustrating the difference here discussed.

### *III. Modification of the Method.*

We had measured in the preceding experiments the rate of diffusion of distilled water under an initial pressure head of about 110 mm. of water against solutions of various electrolytes. When we reverse the procedure, putting the solution of electrolyte inside of the bag and the distilled water outside, the latter will diffuse into the bag and at the same time the solution will commence to diffuse outside. Since, however, the concentration of the solute is at the beginning greater inside than outside, water will at first diffuse into the bag and the liquid in the manometer tube will rise. After 1 hour or more the maximum height in the glass tube is reached and the level of liquid in the glass tube commences to fall again. By comparing the height to which the liquid in the tubes rises in about 70 minutes we shall get not a quantitative measure, but a qualitative indication of the relative influence of the various types of ions on the rate of diffusion of water; for we shall show in the next chapter that the differences observed are the expression of differences in the rate of diffusion of water into the solution and not the expression of differences in the rate of diffusion of the solute into the distilled water. We used  $\text{M}/128$  solutions, since we found that  $\text{M}/64$  and even  $\text{M}/32$  solutions of cane

sugar or dextrose caused only a slight rise in the level of liquid. This excludes the possibility that the rise in liquid observed could be ascribed to the gas pressure of the solute.

A comparison of the relative rise of the liquid in the tube in Table I leads to a verification of the two rules.  $M/128$  solutions of the chlorides of the alkaline metals cause a considerable rise, the rise increasing with an increase in the "radius" of the cation in the order Li, Na, K, Rb (Li having the smallest ionic radius). This agrees with the assumption that water behaves towards these solutions as if its molecules were positively charged and attracted by the anion and repelled by the cation, the repulsion being the greater the smaller the "ionic radius" of the cation.

The most striking fact is that the  $M/128$  solutions of the neutral salts of bivalent metals cause no rise. In fact the water fell constantly in the glass tube containing the solutions, since the initial pressure head in the tube was always about 25 mm. of the column of solution. Hence solutions of  $M/128$   $MgCl_2$ ,  $CaCl_2$ ,  $SrCl_2$ ,  $BaCl_2$ ,  $CoCl_2$ , and  $MnCl_2$  possess actually no attraction for water (beyond that caused by the laws of gas pressure). This was to be expected since the positively charged water molecules are repelled more powerfully by the bivalent than by the univalent cations. This difference between the salts with univalent and bivalent cations exists not only in the case of chlorides but also of nitrates and of sulfates (Table I), and, as we shall see later, the hydroxides. The concentration of all solutions except two used in the table was  $M/128$ . The slight differences in the osmotic pressure of the various solutions do not influence the result, as we shall show in a later chapter.

Table II shows that the attractive action of sodium salts increases with the valency of the anion, as our rule demands. Table I also shows that in the case of neutral salts with univalent and bivalent cations the sulfates attract water more powerfully than the chlorides.

Table I shows, moreover, that the salts with trivalent and tetravalent cations have a very powerful attraction for water, which we should expect if water molecules behave like negatively charged bodies towards solutions of these salts.



TABLE I.

A. Solutions which attract water as if its molecules were positively charged.					
Chlorides.	Level of solution after 70 min.	Nitrates.	Level of solution after 70 min.	Sulfates.	Level of solution after 70 min.
	<i>mm.</i>		<i>mm.</i>		<i>mm.</i>
LiCl.....	96	LiNO <sub>3</sub> .....	64	Li <sub>2</sub> SO <sub>4</sub> .....	356
NaCl.....	106	NaNO <sub>3</sub> .....	112	Na <sub>2</sub> SO <sub>4</sub> .....	410
KBr.....	131				
RbCl.....	154				
CsCl.....	143				
MgCl <sub>2</sub> .....	12	Mg(NO <sub>3</sub> ) <sub>2</sub> .....	10	MgSO <sub>4</sub> .....	74
CaCl <sub>2</sub> .....	18	Ca(NO <sub>3</sub> ) <sub>2</sub> .....	22	CaSO <sub>4</sub> .....	45
SrCl <sub>2</sub> .....	13	Sr(NO <sub>3</sub> ) <sub>2</sub> .....	19		
BaCl <sub>2</sub> .....	16	Ba(NO <sub>3</sub> ) <sub>2</sub> .....	15		
CoCl <sub>2</sub> .....	15				
B. Solutions which attract water as if its molecules were negatively charged.					
Ce <sub>2</sub> Cl <sub>6</sub> .....	260	Ce <sub>2</sub> (NO <sub>3</sub> ) <sub>6</sub> .....	531		
Al <sub>2</sub> Cl <sub>6</sub> .....	376				
Fe <sub>2</sub> Cl <sub>6</sub> .....	663				
m/320 ThCl <sub>4</sub> .....	630	m/320 Th(NO <sub>3</sub> ) <sub>4</sub>	535		

TABLE II.

Solutions which attract water as if its molecules were positively charged.	
	Osmotic pressure in mm. of height of solution after 70 min.
	<i>mm.</i>
NaCl.....	106
NaNO <sub>3</sub> .....	112
Na <sub>2</sub> SO <sub>4</sub> .....	410
Na <sub>2</sub> succinate.....	601
Na <sub>2</sub> tartrate.....	497
NaHCO <sub>3</sub> .....	294
Na <sub>2</sub> CO <sub>3</sub> .....	520
Na <sub>2</sub> HPO <sub>4</sub> .....	484
Na <sub>3</sub> citrate.....	846
K <sub>4</sub> Fe(CN) <sub>6</sub> .....	930

#### IV. *The Rate of Diffusion of Electrolytes through the Collodion Membranes.*

We therefore see that no rise in the manometer occurs when a collodion bag filled with a  $M/128$  solution of  $MgCl_2$  or  $CaCl_2$  is dipped into a beaker with distilled water, while a considerable rise occurs when the bag is filled with a  $M/128$  solution of  $NaCl$  or  $Na_2$  oxalate. The writer was at first inclined to assume that salts of the type of  $MgCl_2$  diffused so rapidly through the collodion membrane that no osmotic effect had time to occur, while the diffusion of  $NaCl$  or  $Na_2$  oxalate was supposed to be much slower. In order to test this idea the following experiments were made.

Various concentrations of  $LiCl$ ,  $NaCl$ ,  $KCl$ ,  $MgCl_2$ , and  $CaBr_2$  were put inside of the collodion bag and the latter was surrounded by a beaker containing 350 cc. of  $H_2O$ . Every half hour the amount of  $Cl$  or  $Br$  contained in 20 cc. of the liquid in the beaker was ascertained by titration after Volhard's method. (Table III.)

TABLE III.

Quantity of 0.1 N  $Cl$  (or  $Br$ ) found in 20 cc. of the outside solution.

After 30 minutes.							
	M/1	M/2	M/4	M/8	M/16	M/32	M/64
	cc.	cc.	cc.	cc.	cc.	cc.	cc.
$LiCl$ .....		5.4	2.5	1.28	0.585	0.265	0.140
$NaCl$ .....		5.5	2.7	1.15	0.635	0.25	0.125
$KCl$ .....	12.8	5.4	3.2	1.45	0.59	0.32	0.20
$MgCl_2$ .....		10.7	5.7	2.3	1.3	0.7	
$CaBr_2$ .....	23.9	11.8	5.5	2.7	1.05	0.52	
After 60 minutes.							
$LiCl$ .....		8.1	4.1	1.9	0.90	0.425	0.22
$NaCl$ .....		7.55	4.2	1.75	0.94	0.38	0.195
$KCl$ .....	15.0	7.6	4.7	2.2	0.95	0.53	0.28
$MgCl_2$ .....	30.4	17.4	9.25	3.6	1.9	1.0	
$CaBr_2$ .....	28.4	14.3	7.3	3.7	1.5	0.8	

These experiments show first that the rate of diffusion of these five salts occurs in proportion to their concentration and second that in the same time almost equal numbers of molecules of LiCl, NaCl, KCl,  $MgCl_2$ , and  $CaBr_2$  diffuse from equally concentrated solutions into the distilled water. (The diffusion velocity of KCl may possibly be slightly larger than that of LiCl or NaCl.)

Hence the fact that the liquid does not rise when the collodion bag is filled with solutions of salts with a bivalent cation while it rises when it is filled with salts with univalent cation of the same osmotic pressure cannot be explained on the assumption that the salts like  $MgCl_2$  or  $CaCl_2$  diffuse out more rapidly into distilled water than do

TABLE IV.

Quantity of 0.1 N HCl and $H_2SO_4$ found in 20 cc. of the surrounding $H_2O$ .											
After 30 minutes.											
	N/1	N/2	N/4	N/8	N/16	N/32	N/64	N/128	N/256	N/512	N/1024
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
HCl.....	14.9	8.4	4.1	1.76	0.795	0.405	0.175	0.09	0.04	0.02	0.01
$H_2SO_4$ .....	15.3	7.1	3.3	1.4	0.77	0.33	0.15	0.06	0.02	0.01	0.01
After 60 minutes.											
HCl.....	20.1	11.1	5.5	2.5	1.2	0.54	0.25	0.13	0.07	0.03	0.02
$H_2SO_4$ .....	18.2	9.4	4.2	2.0	0.99	0.44	0.21	0.10	0.04	0.02	0.01

salts of the type NaCl. The only other possible explanation is that water diffuses more rapidly into a collodion bag filled with a solution of a salt like NaCl than into a bag filled with a salt like  $CaCl_2$  of the same osmotic pressure.

A similar experiment was carried out with the rate of diffusion of HCl and  $H_2SO_4$ . The results are given in Table IV.

The quantities of acid diffusing out are again for each acid approximately in proportion to the concentration of the acid, and almost equal numbers of molecules diffuse out from the same molecular concentrations of the two acids though the rate of diffusion seems to be a trifle less for  $H_2SO_4$  than for HCl. (The reader will notice that the

equal concentrations in Table IV mean equal normality and not equimolecular concentration as in Table III for the neutral salts.)

In order to compare two salts with univalent and bivalent anions,  $M/128$  NaCl and  $M/128$  Na<sub>2</sub> oxalate were chosen, since they vary considerably in their attraction for water. In the  $M/128$  Na<sub>2</sub> oxalate solution the liquid in the manometer rose in 30 minutes to a height of 420 mm. and in the  $M/128$  solution of NaCl to a height of 60 mm. It might again be argued that this difference was due not to a more rapid diffusion of water into the Na<sub>2</sub> oxalate solution but to a more rapid diffusion of the NaCl into the distilled water. Titration showed that after 30 minutes the NaCl solution inside the bag had been diminished by 3.6 cc.  $M/100$  NaCl per 20 cc. solution, while the Na<sub>2</sub> oxalate solution had been diminished by 3.8 cc.  $M/100$  Na<sub>2</sub> oxalate. Again the difference in the rate of diffusion of the two salts is small and cannot explain the large difference in the osmotic behavior of the two salts. We are therefore compelled to ascribe this difference to a difference in the influence of the ions on the rate of the diffusion of water into these solutions, as the rules expressed at the end of Chapter I demand.

While this is correct for solutions of electrolytes above a molecular concentration of  $M/128$ , it is possible that for solutions of electrolytes of a concentration below a certain limit the nature of the electrolyte may modify the result. We intend to deal with this possibility in a later paper.

#### *V. Measurements of the Approximate Value of the Electric Force Influencing Diffusion.*

These experiments show that aside from the gas pressure of water a second type of forces, namely electrical forces, influence the rate of diffusion of water from solvent to solution of electrolyte through a collodion membrane. If we denote these two types of forces by  $p$  and by  $e$  (for the osmotic pressure and the electrical attraction respectively), the question arises whether we cannot arrive at an approximate determination of the value of  $e$ . This can be done in a simple way. When we fill the collodion bag with a solution, e.g. of  $M/128$  KCl, this solution has approximately the same osmotic



pressure as a  $M/64$  solution of cane sugar. If we surround the bag containing the  $M/128$  solution of KCl with a solution of  $M/64$  cane sugar approximately equal numbers of molecules of water should impinge on both sides of the collodion membrane and no change in the level of the two liquids should occur, if only the osmotic pressure  $p$  were active. As a matter of fact the water will diffuse from the  $M/64$  sugar solution into the  $M/128$  solution of KCl owing to the electrical force  $e$  due to the electrolyte. By using higher concentrations of cane sugar we arrive at finding one concentration which balances the combined osmotic pressure and electrical force of a  $M/128$  solution of NaCl. The concentration of cane sugar which balances the combined osmotic and electric attraction of  $M/128$  NaCl for water was found to be approximately  $M/8$ . Assuming that the osmotic attraction  $p$  of  $M/128$  NaCl for water is equal to the osmotic pressure of a  $M/64$  solution of cane sugar, the force  $e$  of the  $M/128$  KCl solution must be equal to the osmotic pressure  $M/8 - M/64 = 7 M/64$ , or  $\frac{7 \times 22.4}{64} = 2.4$  atmospheres. In this way we have determined the value of  $e$  for a number of electrolytes by selecting as the balancing concentration that concentration of a cane sugar solution in which the level of liquid does not rise when a beaker filled with the cane sugar solution is dipped into a solution of the electrolyte. This method has the advantage that the concentration of the balancing solution of cane sugar can be roughly estimated in about 5 minutes, although in fact most of our experiments were continued for half an hour or an hour. We used the same set of membranes for a large number of experiments and two sets were used in all; occasionally a membrane began to leak and we had to replace it by another membrane. Measurements of the height of the column of solution inside the tube were taken at first every 5 and later every 10 minutes. The sugar solutions were in the bag and 350 cc. of the  $M/128$  NaCl solution were outside. The initial level of the liquid in the manometer tube was about 25 mm. above the level of the sugar solution. Table V gives the results of our measurements. The balancing solution is always that concentration of cane sugar in which the level of liquid in the manometer tube falls slightly in the first 10 minutes, while in the next higher concentration of cane sugar a marked rise occurs. It

should, however, be stated that this sharp line of demarcation was not found to exist in all solutions. As a consequence the figures of this table serve mainly the purpose of confirming the two rules.

The first vertical column gives the molecular concentration of the solution of the electrolyte which was in every case selected so that on the assumption of complete electrolytic dissociation the solution was isosmotic to a  $M/64$  solution of cane sugar. These solutions were, of course, not strictly isosmotic but we shall see in Chapter VIII that such small differences in osmotic pressure as occurred in these experiments cannot influence the result, on account of the fact that the maximal value of the electric force is generally reached in concentrations of the electrolytes lower than those used in this table and that a further rise in concentration of the electrolyte does not materially affect the value of  $e$ . For this reason we must not deduct  $\frac{1}{64}$  from the concentration of the balancing solution in order to get the value of  $e$ , but a much smaller figure which will fall within the limits of the exactness of our methods. Therefore, the concentration of the balancing solution of cane sugar can be considered as a rough approximation of the value of  $e$ .

The second vertical column gives the nature of the electrolyte, the third the pH of the solution, and the fourth the approximate molecular concentration of the *balancing* sugar solution. The fifth column gives the value  $e$  in terms of atmospheres of the balancing solution. All the values of the balancing concentration are only rough approximations.

These data find their full explanation on the basis of the two rules expressed at the end of Chapter I.

First we notice that the electrolytes of the first group (*A*, in Table V) possessing univalent or bivalent cations attract water as if the molecules of the water were positively charged bodies. Thus the concentration of the balancing solution (and the value of  $e$ ) increases with the increase in the number of charges of the anion and diminishes with an increase in the number of charges of the cation—the latter acting as if it repelled the positively charged water molecules.

We also notice that in Group *A* the repelling action of the cation increases inversely with its "atomic radius," the lithium salts having a lower balancing concentration and a lower value of  $e$  than the sodium or potassium salts with the same anion.

It is especially worth mentioning that the hydroxides act exactly like the chlorides or nitrates, thus disposing of the possibility that the hydroxyl ions occupy a privileged position in the electrification of water.

TABLE V.

*Approximate Concentration of Balancing Solution of Cane Sugar for Various Solutions of Electrolytes.*

A. Electrolytes towards which water acts as if it were positively charged.					
Molecular concentration.	Nature of electrolyte.	pH	Approximate molecular concentration of balancing solution of cane sugar.		Approximate value of $\epsilon$ in atmospheres.
M/128	LiCl.....	5.4	Slightly above	M/32	Slightly above 0.7
M/128	NaCl.....	5.4		M/8	2.8
M/128	KCl.....	5.3		M/8	2.8
M/128	RbCl.....	5.5		M/4	5.6
M/192	MgCl <sub>2</sub> .....	5.4		M/64	About 0
M/192	Mg(NO <sub>3</sub> ) <sub>2</sub> .....	5.6	Below	M/64	" 0
M/192	CaCl <sub>2</sub> .....	5.5	"	M/64	" 0
M/192	SrCl <sub>2</sub> .....	5.4	"	M/64	" 0
M/192	BaCl <sub>2</sub> .....	5.4		M/64	" 0
M/192	CoCl <sub>2</sub> .....	5.0	Below	M/64	" 0
M/192	MnCl <sub>2</sub> .....	5.3	"	M/64	" 0
M/128	LiOH.....			M/16	1.4
M/128	NaOH.....			3M/32	2.1
M/128	KOH.....			M/4	5.6
M/192	Ca(OH) <sub>2</sub> .....		Less than	M/64	About 0
M/192	Ba(OH) <sub>2</sub> .....		" "	M/64	" 0
M/128	NH <sub>4</sub> OH.....	10.2		M/32	0.7
M/192	Li <sub>2</sub> SO <sub>4</sub> .....	5.4		M/4	5.6
M/192	Na <sub>2</sub> SO <sub>4</sub> .....	5.4	Slightly above	M/4	Slightly above 5.6
M/192	K <sub>2</sub> SO <sub>4</sub> .....	5.4	Less than	M/2	" below 11.2
M/128	MgSO <sub>4</sub> .....	5.4		M/32	0.7
M/128	CaSO <sub>4</sub> .....	5.4		M/16	1.4
M/128	MnSO <sub>4</sub> .....	5.4	Slightly less than	M/32	Slightly below 0.7
M/128	KCNS.....	5.5		M/8	2.8
M/128	K acetate.....	6.3	Slightly above	M/4	Slightly above 5.6
M/192	K <sub>2</sub> tartrate.....	6.4	" "	M/2	" " 11.2
M/192	K <sub>2</sub> oxalate.....	5.9		M/2	11.2
M/256	K <sub>2</sub> HPO <sub>4</sub> .....	8.2	Slightly above	M/2	Slightly above 11.2
M/256	K <sub>3</sub> citrate.....	7.8	" "	3M/4	" " 16.8
M/320	K <sub>4</sub> Fe(CN) <sub>6</sub> .....	6.2	" below	3M/4	" below 16.8

TABLE V—*Concluded.*

B. Electrolytes towards which water acts as if it were negatively charged.				
Molecular concentration.	Nature of electrolyte.	pH	Approximate molecular concentration of balancing solution of cane sugar.	Approximate value of $\epsilon$ in atmospheres.
M/192	BeCl <sub>2</sub> .....	4.5	M/8	2.8
M/512	Ce <sub>2</sub> Cl <sub>6</sub> .....	5.1	M/4	5.6
M/512	La <sub>2</sub> Cl <sub>6</sub> .....	5.2	Slightly above 3M/8	Slightly above 8.4
M/512	Al <sub>2</sub> Cl <sub>6</sub> .....	3.8	" " M/2	" " 11.2
M/512	Cr <sub>2</sub> (NO <sub>3</sub> ) <sub>6</sub> .....		" less than M/2	" below 11.2
M/320	ThCl <sub>4</sub> .....	3.2	" above 3M/8	" above 8.4
M/128	BeSO <sub>4</sub> .....	3.5	M/64	0.3
M/320	Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> .....	3.8	M/8	2.8
M/320	Cr <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> .....		M/32	0.7
M/128	HCl.....		M/32	0.7
M/128	HNO <sub>3</sub> .....	2.1	M/64	0.3
M/128	Acetic acid....	3.3	Less than M/64	About 0
M/192	H <sub>2</sub> SO <sub>4</sub> .....		" " M/64	" 0
M/192	Oxalic acid....	2.2	" " M/64	" 0
M/192	Tartaric acid...	2.6	" " M/64	" 0
M/256	Citric acid....	3.0	" " M/64	" 0
M/256	H <sub>3</sub> PO <sub>4</sub> .....	2.4	" " M/64	" 0

The electrolytes of Group *B*, including all salts with trivalent and tetravalent cations, influence the rate of diffusion of water as if the water molecules were negatively charged, being attracted by the cation and repelled by the anion. The correctness of this assumption lies in the fact that the attraction of this group for water is greater when the anion is univalent than when it is bivalent, the repelling action being greater in the latter case. Thus the value for  $\epsilon$  is in Group *B* greater when the anion is a chloride than when it is a sulfate, while in the preceding group of electrolytes towards which water was positively charged the sulfates acted more strongly than the chlorides or nitrates or hydroxides.

The acids belong to Group *B*, although the hydrogen ion carries only one charge. But the latter fact is compensated by the smallness of the "ionic radius" of hydrogen which is the smallest of all elements. This smallness of the "ionic radius" makes the electrostatic action of the positively charged nucleus comparatively strong. Li comes next among the univalent cations in smallness of "ionic radius"



and this explains why the lithium salts attract water less actively than the sodium salts with the same anion.

Since the solutions of some of the salts with trivalent and tetravalent cations are acid (due to hydrolysis), it might be argued that it is only the presence of free acid which makes the salts of Group *B* behave differently from those of Group *A*. This assumption is disproved by the fact that  $\text{La}_2\text{Cl}_6$  and  $\text{Ce}_2\text{Cl}_6$  which belong to Group *B* are neutral salts; *i.e.*, the hydrogen ion concentration of their solution has a pH of the order of that of the solution of neutral salts of Group *A*.

Since, however, the idea of a specific rôle of hydrogen and hydroxyl ions is so general in the colloid literature it seemed of importance to make sure that the differences we have established between the salts

TABLE VI.

Nature of solution.	pH	Concentration of balancing cane sugar solution.
M/512 $\text{La}_2\text{Cl}_6$ .....	5.2	Slightly above 3M/8
M/512 $\text{Ce}_2\text{Cl}_6$ .....	5.15	“ below 3M/8
M/192 $\text{MgCl}_2$ .....	5.1	Less than M/64
M/128 $\text{MgSO}_4$ .....	5.05	M/64
M/128 $\text{KCl}$ .....	5.0	M/16
M/192 $\text{K}_2\text{SO}_4$ .....	5.0	3M/16

of Groups *A* and *B* remain valid when the salts have an identical hydrogen ion concentration. The pH of M/512  $\text{La}_2\text{Cl}_6$  was found to be 5.2, that of M/512  $\text{Ce}_2\text{Cl}_6$  5.1 (due to the presence of  $\text{CO}_2$ ).<sup>7</sup> We prepared solutions of M/128  $\text{KCl}$ , M/192  $\text{MgCl}_2$ , M/192  $\text{K}_2\text{SO}_4$ , and M/128  $\text{MgSO}_4$  of the same or even a slightly lower pH, namely 5.0 to 5.1. Table VI gives the balancing concentration of cane sugar for the six solutions.

We notice, first, that for the same pH of about 5.1 the salts with bivalent cation show the characteristic low value for  $e$ , their attraction for water being less than that of the salts of K as well as of La or Ce.

<sup>7</sup> The pH of the distilled water used varied between 5.1 and 5.3 and this accounts for the pH found in our solutions of neutral salts.

We notice, second, that the attraction for water is greater in the case of  $\text{MgSO}_4$  than in the case of  $\text{MgCl}_2$ , and greater in the case of  $\text{K}_2\text{SO}_4$  than in the case of  $\text{KCl}$ , showing that up to  $\text{pH} = 5.0$  or  $5.1$  these salts attract positively charged water molecules. Hence the action of the salts of Group *B* is due to the trivalent or tetravalent cation and the  $\text{H}$  ion owes its effect not to any specific "adsorption" but to the smallness of its "atomic radius," which gives it a high electrostatic effect.

On the other hand, a high degree of acidity of a solution of a salt with a bivalent cation induces a negative charge in the water molecule. This is true for beryllium salts. In this case the action of  $\text{H}$  ions is simply added to that of the  $\text{Be}$  ions (which have a small "ionic ra-

TABLE VII.

Nature of solution.	pH	Concentration of balancing cane sugar solution.
M/192 $\text{Na}_2\text{CO}_3$ .....	9.2	Slightly above M/2
M/192 $\text{NaHCO}_3$ .....	11.0	" " 3M/8
M/256 $\text{Na}_3\text{PO}_4$ .....	12.0	" " M/2
M/256 $\text{Na}_2\text{HPO}_4$ .....	8.6	" " M/2
M/256 $\text{NaH}_2\text{PO}_4$ .....	4.2	M/16
M/256 $\text{H}_3\text{PO}_4$ .....	2.4	Less than M/64

dus"), and the combined effect of both ions exceeds the effect of the  $\text{Cl}$  ions. We also notice that  $\text{BeSO}_4$  acts less powerfully than  $\text{BeCl}_2$ , as it should if in the presence of these salts water is negatively charged. The  $\text{Be}$  salts therefore belong in Group *B*. We shall come back to the effects of a mixture of two electrolytes in another paper.

From what has been said it is to be expected that the substitution of an  $\text{H}$  for an  $\text{Na}$  ion in the carbonates or phosphates increases the cation effect owing to the fact that  $\text{H}$  has a smaller "ionic radius" than  $\text{Na}$  (Table VII).

It is obvious that in the two carbonates and the first three phosphates toward which (as we shall see in the next chapter) water is positively charged, the attraction of the  $\text{CO}_3$  or  $\text{PO}_4$  for water diminishes the more the more  $\text{H}$  ions replace the  $\text{Na}$  ions, as our theory demands.

*VII. Direct Proof of the Influence of the Valency and Radius of Ions on the Sign of the Charge of Water.*

Experiments on the migration of water through a collodion membrane under the influence of a constant current (electric endosmose) furnish the direct proof that our assumption is correct.

It is known that if a current is sent through the walls of a porous cell bounded on both sides by the same liquid conductor, water migrates either to the cathode or the anode. This migration is indicated by a rise in the level of the water on the side of that electrode towards which the water migrates. Helmholtz explained this phenomenon by the formation of an electrical double layer at the boundary of the diaphragm and the water particles. When a current is sent through the liquid the positively charged particles of water will move towards the cathode and if the water particles in contact with the wall are negatively charged they will move towards the anode. On account of the internal friction of the liquid the neighboring layers of liquid will be dragged along with the charged particles. This causes the rise of the liquid on the side of that electrode towards which the particles move.<sup>8</sup>

This electric osmose gives us a chance to test our assumption that the water molecules assume a different charge according to the nature of the electrolyte; namely, (1) that the water molecules assume a positive charge when the collodion bag is filled with solutions of neutral salts with a monovalent or bivalent cation or with solutions of hydroxides with monovalent or bivalent cations, (2) that the water molecules assume a negative charge when the collodion bag is filled with solutions of neutral (or acid) salts with a trivalent or tetravalent cation, or with solutions of acids.

If our assumption is correct, water should move towards the cathode in the case of the electrolytes mentioned in (1) (Group A, Table V) and towards the anode in the case of electrolytes mentioned in (2) (Group B, Table V). This is actually the case.

The collodion bags, closed by a perforated rubber stopper through the hole of which a glass tube with a bore of 2 mm. was inserted,

<sup>8</sup> It should be noticed that a collodion membrane belongs in all probability to the type of non-porous diaphragms.

were filled with a dilute solution of the electrolyte the influence of which on the electrification of water was to be tested. The collodion bag was suspended in a beaker containing the same solution of the electrolyte as the bag, the two solutions being identical in every respect, nature of solute, concentration, and pH. By pushing the glass tube (manometer) a little deeper into the bag the liquid was adjusted so that it rose in the glass tube about 25 mm. above the level in the beaker. One electrode (platinum wire) was dipped into the glass tube, another into the beaker, and a constant current was sent through with an intensity of about 4.8 milliamperes. This intensity was kept constant whenever possible. The level of the liquid in the glass tube was measured at the beginning of the experiment and it was easy to determine the sign of the electrification of the water molecules by observing whether the current caused the liquid in the glass tube to rise or to fall. When the anode was in the glass tube and the water rose we could be sure that the water was negatively charged, and when the level of the water fell rapidly under such conditions in the tube, we could be sure that the water was positively charged. When it fell very slowly the fall might have been due to the pressure head in the glass tube. A reversal of the direction of the current removed any doubt that might arise. The experiments gave a complete confirmation of the assumption made in the two rules.

It was found that in the presence of solutions of neutral salts belonging to Group A (Table V), *i.e.* salts or hydroxides with univalent cations, the water migrated to the cathode and hence was positively charged, as our theory demands. The following salts were tried: M/1000 and M/500 LiCl, M/1000 and M/500 Li<sub>2</sub>SO<sub>4</sub>, M/256 NaCl, M/1000 Na<sub>2</sub> oxalate, M/128 Na<sub>2</sub>SO<sub>4</sub>, M/256 Na acetate, M/256 LiOH, M/256 NaOH, M/1000 KCl, M/512 K<sub>2</sub> oxalate, M/1024 K<sub>3</sub> citrate, and M/1000 K<sub>4</sub>Fe(CN)<sub>6</sub>.

In solutions of the neutral salts with bivalent cation, M/512 MgCl<sub>2</sub>, CaCl<sub>2</sub>, SrCl<sub>2</sub>, and BaCl<sub>2</sub>, water migrated neither to the anode nor to the cathode; these salts did not cause, or only to a very slight degree, an electrification of the water molecules as was to be expected. M/512 CaSO<sub>4</sub> on the other hand, induced a slight migration of water to the cathode, as our theory demanded.



In the presence of electrolytes belonging to Group *B* (Table V) water migrated to the anode, as it should on the basis of our rules. The following electrolytes were tried: M/1024  $\text{La}_2\text{Cl}_6$ , M/1024 lanthanum ammonium nitrate, M/1024  $\text{Ce}_2\text{Cl}_6$ , M/1024  $\text{Al}_2\text{Cl}_6$ , M/1024  $\text{Al}_2(\text{SO}_4)_3$ , and M/1024  $\text{ThCl}_4$ .

In the presence of M/250 HCl and M/250  $\text{HNO}_3$ , tartaric and M/512 phosphoric acids water also migrated to the anode, but the rate of migration was very slow; in the case of M/500 or M/1000  $\text{H}_2\text{SO}_4$  practically no migration was noticeable.

In the presence of M/512  $\text{BeCl}_2$  water migrated also to the anode as was to be expected. Most interesting were the experiments with M/500  $\text{H}_3\text{PO}_4$ ,  $\text{NaH}_2\text{PO}_4$ ,  $\text{Na}_2\text{HPO}_4$ , and  $\text{Na}_3\text{PO}_4$ . All, with the exception of  $\text{H}_3\text{PO}_4$ , caused a migration to the cathode, which again is in agreement with our rule. It may also be stated that when M/512 NaCl solution was rendered acid, *e.g.* when the pH was 4.0 or 3.7, the water migrated to the anode.

We may therefore state that our assumption concerning the influence of electrolytes on the sign of the electrical charge of water molecules is supported not only by the facts for the explanation of which it was made but also by the direct experiments with electrical osmose.

#### *VIII. Influence of the Concentration of the Electrolyte in Solution on the Electric Effect.*

When water diffuses from pure solvent to a solution, the initial rate of diffusion seems to vary approximately with the concentration of the solute, as long as the latter is a non-electrolyte. When it is an electrolyte the laws of gas pressure are not suspended but may be masked by the electrical forces which act simultaneously with the laws of gas pressure. It seemed of interest to determine the influence of the concentration of the electrolyte upon the rate of diffusion of water through the collodion membrane. To accomplish this the balancing solution of cane sugar was determined for various concentrations of the electrolyte. Table VIII gives the results of such measurements for various concentrations of KCl, of  $\text{Na}_2\text{SO}_4$ , and of  $\text{MgCl}_2$ .

The effects observed in the solutions of  $\text{MgCl}_2$  may be only osmotic pressure effects with little or no influence of an electrical character.

In the case of  $\text{KCl}$  and of  $\text{Na}_2\text{SO}_4$  it seems that the maximal effect of  $e$  is reached at a very low concentration of the electrolyte, near  $m/320$  in the case of  $\text{KCl}$  and near  $m/960$  in the case of  $\text{Na}_2\text{SO}_4$ . A further increase of the concentration to 100 times or more caused no further increase in the value of the balancing concentration. This would be intelligible on the assumption that the electrical effect is of the order of a saturation phenomenon; *e.g.*, the saturation of the membrane with electrolyte. Following ideas suggested in Langmuir's papers,<sup>9</sup> we may assume that electrolyte, collodion, and water ar-

TABLE VIII.

Molecular concentration of $\text{KCl}$ .	Concentration of balancing cane sugar solution.	Molecular concentration of $\text{Na}_2\text{SO}_4$ .	Concentration of balancing cane sugar solution.	Molecular concentration of $\text{MgCl}_2$ .	Concentration of balancing cane sugar solution.
$m/2560$	$m/64$	$m/15,360$	$m/64$	$m/192$	0
$m/1280$	$m/64$	$m/7680$	$m/16$	$10m/192$	0
$m/960$	$m/32$	$m/3840$	$3m/32$	$25m/192$	0
$m/640$	$m/16$	$m/1920$	$m/8$	$50m/192$	Slightly above $3m/32$
$m/320$	$m/8$	$m/960$	$m/4$	$100m/192$	$3m/16$
$m/128$	$m/8$	$10m/192$	$m/4$	$150m/192$	$3m/8$
$10m/128$	$m/8$	$50m/192$	$3m/16$	$200m/192$	About $m/2$
$100m/128$	$m/8$				

range themselves in the collodion membrane in a definite pattern or space-lattice held together by chemical forces very much as the particles in a crystal, with this difference only that the molecules of water and the ions of the salts in the pattern have the degree of mobility characteristic of liquids. The electric fields created at the two surfaces of the membrane determine the charge of the particles of water impinging upon or lying adjacent to the surface of the membrane. On the solution side the membrane is bounded by the ions of the solute while on the side of pure solvent it is bounded by water alone and this difference determines the direction of the

<sup>9</sup> Langmuir, I., *J. Am. Chem. Soc.*, 1916, xxxviii, 2221.

diffusion under the influence of these forces, which may be electrostatic in character.

It follows from the data of Table VIII that the balancing concentrations given in Table V are the maximal values for  $e$  for each of the electrolytes mentioned and that it is not necessary to deduct  $\frac{1}{64}$  from this value. The real deduction to be made is so small that it falls within the limits of error of the determination of the balancing concentration and the latter may therefore be taken as the rough expression for the value of the electrical forces.

#### SUMMARY.

1. When pure water is separated by a collodion membrane from a watery solution of an electrolyte the rate of diffusion of water is influenced not only by the forces of gas pressure but also by electrical forces.

2. Water is in this case attracted by the solute as if the molecules of water were charged electrically, the sign of the charge of the water particles as well as the strength of the attractive force finding expression in the following two rules. (a) Solutions of neutral salts possessing a univalent or bivalent cation influence the rate of diffusion of water through a collodion membrane, as if the water particles were charged positively and were attracted by the anion and repelled by the cation of the electrolyte; the attractive and repulsive action increasing with the number of charges of the ion and diminishing inversely with a quantity which we will designate arbitrarily as the "radius" of the ion. The same rule applies to solutions of alkalis. (b) Solutions of neutral or acid salts possessing a trivalent or tetravalent cation influence the rate of diffusion of water through a collodion membrane as if the particles of water were charged negatively and were attracted by the cation and repelled by the anion of the electrolyte. Solutions of acids obey the same rule, the high electrostatic effect of the hydrogen ion being probably due to its small "ionic radius."

3. The correctness of the assumption made in these rules concerning the sign of the charge of the water particles is proved by experiments on electrical osmose.

4. A method is given by which the strength of the attractive electric force of electrolytes on the molecules of water can be roughly estimated and the results of these measurements are in agreement with the two rules.

5. The electric attraction of water caused by the electrolyte increases with an increase in the concentration of the electrolyte, but at low concentrations more rapidly than at high concentrations. A tentative explanation for this phenomenon is offered.

6. The rate of diffusion of an electrolyte from a solution to pure solvent through a collodion membrane seems to obey largely the kinetic theory inasmuch as the number of molecules of solute diffusing through the unit of area of the membrane in unit time is (as long as the concentration is not too low) approximately proportional to the concentration of the electrolyte and is the same for the same concentrations of  $\text{LiCl}$ ,  $\text{NaCl}$ ,  $\text{MgCl}_2$ , and  $\text{CaCl}_2$ .





## A STUDY OF THE FAT METABOLISM OF INFANTS AND YOUNG CHILDREN.

### I. FAT IN THE STOOLS OF BREAST FED INFANTS.

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In recent years the examination of infants' stools, on the assumption that they represent the completeness of digestion and absorption of the different food constituents, has become a matter of great importance in the study of infant nutrition. Especially is this true in regard to the fat of the stool. It is generally believed that a knowledge of the fat content of stools and the distribution of the fat as soap, free fatty acids and neutral fat is highly desirable from a practical point of view. This knowledge is usually obtained by a superficial clinical examination of the stool and conclusions are frequently drawn from such findings which seem to us to be of questionable value.

There is, however, available in the literature a considerable amount of material which has been obtained by more complete and accurate chemical investigations. For various reasons these findings have not established a definite standard of the composition of infants' stools under varying conditions. In the first place, no very large number of stools has been reported by the same investigator. Moreover, the use of different methods for fat determination interferes with the correlation of the results obtained by various authors. Another difficulty arises from the fact that the greater part of these results have been obtained in Germany, where living conditions and methods of feeding are different from our own.

Reliable figures on the composition of stools of breast fed infants are particularly scanty. Both for this reason and because of the great importance attached to all information concerning the results of breast feeding, it has seemed fitting to begin a general study of the fat

metabolism of infants and young children with the examination of a considerable number of stools of breast fed infants. The observations were made with a view to answering the following questions:

1. What is normally the per cent. of total fat and what is the distribution of fat as soap, free fatty acids and neutral fats in the stools of healthy breast fed infants?

2. What is the difference in fat content and distribution of fat between normal and abnormal stools?

3. Does the amount and distribution of fat in the stools of healthy breast fed infants vary with the per cent. of fat in the milk and with the amount of fat intake?

The material examined consists of forty-eight collections of feces from thirty-four different infants from 10 days to 10 months of age.

Thirty of these infants were well nourished and, with few exceptions, gaining normally. In the greater number of these cases the appearance of the stools was that typical of normal breast fed infants, largely yellow, granular or pasty, very acid to litmus, with acid aromatic, not unpleasant odor. One child had stools of a peculiar dark orange color. Several of the stools showed more or less mucus and some were partly green in color. A number of the children had distinctly abnormal stools, markedly green in color and containing much mucus.

Four of the children were acutely ill. One of these, so ill that he died two days later, had stools typically normal in appearance, although the daily amount was unusually large. The others had abnormal stools—green, watery with much mucus.

The collected stools were dried quickly on the waterbath to constant weight and ground to a powder. Analyses were made for total fat and its distribution as soap, free fatty acids and neutral fat, according to the method heretofore described by us.<sup>1</sup>

The stool findings for the normal children have been arranged in three groups, according to the gross appearance of the stools. The first group, Table 1, includes the analyses of the stools which were typically normal in appearance, showing neither green color nor evident mucus. Two of the infants were under 10 days old, the milk being colostrum.

1. Holt, Courtney and Fales: *Am. J. Dis. Child.* **17**: 38 (January) 1919.

It is interesting to find that in the normal stools of nursing infants the total fat forms so large a percentage of the dried weight. The average in this table is 42.1 per cent., and there are five instances in which the fat is over 50 per cent. of the total dried weight. This is considerably higher than we have generally found in the case of

TABLE 1.

*Total Fat and Fat Distribution in Typically Normal Yellow Stools.*

Case	Age	Fat per Cent. of Dried Weight	Per Cent. of Total Fat as			Per Cent. Fat in Mother's Milk	Remarks
			Soap	Free Fatty Acids	Neutral Fat		
1. P. L.	4	6 wk.	38.7	74.8	16.6	8.6	
2. P. L.	5	9 wk.	29.3	74.0	14.6	11.4	3.8 Had also 4 tsp. dried milk at 1 feeding
3. W. C.		3 mo.	26.3	70.9	16.9	12.2	2.5
4. P. L.	2	3 wk.	52.0	67.7	24.4	7.9	5.6
5. M. E.		5 mo.	53.1	67.5	19.6	12.9	6.4
6. P. L.	3	3.5 wk.	41.0	63.8	25.6	10.6	4.1 Milk showed 5.5% sugar and 1.5% protein
7. L. H.		3 mo.	29.4	60.9	24.3	14.8	1.8
8. B. T.	1	3 wk.	43.2	57.5	23.1	19.4	
9. E. M.	1	10 wk.	32.7	56.9	21.7	21.4	5.8
10. B. G.	1	6 mo.	43.6	53.4	32.7	13.9	2.6
11. B. G.	3	7 mo.	57.7	52.4	30.6	17.0	
12. P. L.	1	10 da.	55.6	46.8	35.5	17.7	... Milk colostrum; a little mucus in stool
13. B. T.	2	3.5 wk.	43.7	44.5	32.3	23.2	4.3 Milk showed 6.8% sugar and 1.4% protein
14. B. G.	2	6.5 mo.	33.7	40.8	35.5	23.7	
15. B. M.		8 da.	51.5	34.8	41.8	23.4	... Milk colostrum
Average. ....		42.1	57.8	26.3	15.9		

infants with normal digestion fed on dilutions of cow's milk. The findings as regards the distribution of fat are equally striking. The fat which is soap is high in this group, averaging 57.8 per cent. of the total fat. The neutral fat is rather low, averaging 15.9 per cent. of the total fat. The soap predominates over the other forms of fat in every case but one—an infant receiving colostrum.



The second group, Table 2, shows the findings in good stools which were not quite normal in appearance; that is, they were partly green in color or showed some mucus, or had both these characteristics.

TABLE 2.

*Total Fat and Fat Distribution in Good Stools, Not Quite Normal, That Is, Partly Green or Showing Some Mucus.*

Case	Age	Fat per Cent. of Dried Weight	Per Cent. of Total Fat as			Per Cent. Fat in Mother's Milk	Remarks
			Soap	Free Fatty Acids	Neutral Fat		
16. B. N.	7 wk.	36.0	49.6	37.5	12.9		
17. D. T.	6 wk.	61.1	47.2	35.8	17.0	2.5	
18. V. V.	5 wk.	43.9	46.0	41.1	12.9	...	Child not in good condition*
19. V. R.	3 mo.	30.8	45.2	(No separation)		3.9	Child not gaining at time
20. N. K. 2	7 wk.	18.6	41.4	35.1	23.5	2.5	
21. M. C.	8 wk.	10.7	39.7	49.5	10.8	3.3	Stool slightly fermentative
22. M. A.	4 wk.	35.7	38.3	44.6	17.1	5.6	Child not in good condition*
23. J. K.	5 mo.	32.9	36.4	46.5	17.1	6.4	
24. V. H.	9 wk.	41.3	32.9	47.1	20.0	2.0	
25. B. L.	8 wk.	44.9	31.2	55.2	13.6	5.4	
26. L. A.	5 wk.	45.4	29.0	58.0	13.0	1.4	
27. N. K. 1	6 wk.	27.3	27.5	55.8	16.7	2.5	Stools slightly fermentative
28. B. B.	11 wk.	23.3	21.4	45.6	33.0	3.0	
29. R. V.	2 mo.	43.5	20.0	53.7	26.3	5.6	Child not gaining
30. B. W.	4 wk.	38.2	19.3	51.7	29.0	2.6	
31. B. B.	.....	52.3	16.8	50.0	33.2		
Average.....		36.6	33.1	47.2	19.7		

\* These were normal infants but they were suffering from slight temporary indisposition.

In this group the total fat per cent. is somewhat lower than in the first group, the average being 36.6 per cent. of the dried weight; there are only two instances in which it is over 50 per cent., while in two it is under 20 per cent. A marked contrast between this table and the preceding appears in the change in the proportions of free fatty acids

and soap fat. In the second group the fatty acids are high, averaging 47.2 per cent. of the total fat, while the soap average is only 33.1 per cent. In two-thirds of the cases the fatty acid is the predominating form in which the fat is found in the stool. In this group the neutral

TABLE 3.

*Total Fat and Fat Distribution in Stools Which Were Entirely Green or Contained Much Mucus.*

Case	Age	Fat per Cent. of Dried Weight	Per Cent. of Total Fat as			Per Cent. Fat in Mother's Milk	Remarks
			Soap	Free Fatty Acids	Neutral Fat		
32. H. S. 4	11 wk.	18.1	51.9	37.4	10.7		
33. J. F.	10 mo.	12.4	51.6	24.5	23.9	3.8	Child in excellent condition; sugar in milk, 6.6%
34. B. S.	10 wk.	4.4	49.9	40.0	10.1	4.9	Child gaining; much mucus in stool
35. H. S. 3	10 wk.	35.0	45.2	35.6	19.2	...	Chalk mixture for 10 days preceding
36. B. T.	6 wk.	31.7	44.3	34.3	21.4	4.4	Much mucus in stool
37. H. S. 5	12 wk.	21.4	39.2	43.3	17.5	...	Chalk mixture for week preceding
38. E. M. 2	12 wk.	8.8	37.0	30.0	33.0	5.8	Stool normal except for much mucus
39. E. M.	3 mo.	38.3	36.8	31.3	31.9	3.9	Stool very green
40. J. W.	7 mo.	14.1	34.4	35.7	29.9	...	Child gaining slowly; milk scanty
41. J. M.	4 mo.	14.4	34.2	34.3	31.5	1.0	Child gaining slowly; sugar in milk, 5.4%
42. H. S. 2	8 wk.	18.2	27.8	23.9	48.3	...	Stool extremely acid with much mucus
43. P. W.	9 wk.	40.3	26.0	41.9	32.1	...	Stool very green with much mucus
44. H. S. 1	5 wk.	32.3	12.3	64.7	23.0	3.4	Stool extremely acid with much mucus
Average.....		23.0	37.7	36.7	25.6		

fat averages somewhat higher than in the preceding group, it being 19.7 per cent. of the total fat.

Table 3 presents the analyses of a group of stools which might be considered distinctly abnormal; that is, entirely green or containing much mucus.

The most striking feature in this table is the low per cent. of total fat, averaging 23.0 per cent. of the dried weight. In more than half the cases it is below 20 per cent. Since many of these stools consisted very largely of mucus, it is probable that the low fat per cent. is thus to be explained. The two very low figures for total fat in Table 2 may be accounted for in the same way. This type of stool is usually an indication of an abnormal intestinal condition, and under such circumstances the total daily excretion of feces is considerably increased over the normal. Hence the total daily loss of fat would be greater than occurred in children with normal stools in which the per cent. of fat is higher.

When the distribution of the fat in Table 3 is considered, it is seen that the per cent. of neutral fat is much higher than in the other two groups, averaging 25.6 per cent. of the total fat. The average per cent. of the soap and that of the fatty acids are about the same.

TABLE 4.  
*Average Analyses of Stools.*

Fat of Stool, per Cent. of Dried Weight	Per Cent. of Total Fat as		
	Soap	Free Fatty Acids	Neutral Fat
34.5	43.1	36.7	20.2

The five stools in which soap predominated contained much mucus, and all but one were otherwise normal in appearance.

The case, H. S., was followed for some weeks, five stools being examined. The child was well nourished and gaining normally, but had extremely acid stools, with considerable mucus, and she had a severe excoriation of the buttocks. An effort was made to reduce the acidity of the stools by the administration of chalk mixture. Observations 44 and 42 were made before any chalk mixture was given and show very low soap per cent. Before Observations 35 and 37 the child received an ounce of chalk mixture daily for over a week, with the result that the soap fat of the stools was markedly increased. Although no chalk mixture was given just before Observation 32, the effect of the preceding administration seems to have persisted.

The average of the analyses of the stools of all the children in good condition, 44 observations, included in the three tables just discussed, is as shown in Table 4.

This may be taken to represent an average stool of the breast fed infant in good condition.

It must be borne in mind that this average includes the analyses of not only the typically normal stools, but others not quite normal and some that were markedly abnormal in appearance. It is the more striking, therefore, that the fat constitutes over one-third of the dried weight and that the soap is over 40 per cent. of the total fat. Even when allowance is made for an increase in the proportion of soap which takes place during drying, the average soap fat is found to form at least one-third of the total fat of the stool.

TABLE 5.

*Total Fat and Fat Distribution in Stools of Sick Infants.*

Case	Age	Fat per Cent. of Dried Weight	Per Cent. of Total Fat as			Per Cent. Fat in Mother's Milk	Remarks
			Soap	Free Fatty Acids	Neutral Fat		
45. J. D.	8 mo.	57.1	11.0	56.3	32.7	3.4	Ileocolitis, scurvy; stool normal in appearance
46. H. F.	6 mo.	53.7	6.6	49.6	43.8	4.5	Eczema, diarrhea; stool green, watery, fat curds, mucus
47. C. V.	5 mo.	17.2	20.5	(No separation)		0.9	Eczema, erysipelas, diarrhea; stool green, watery
48. J. B.	7 mo.	17.0	29.2	42.7	28.1	...	Syphilitic, temporarily very ill from reaction after salvarsan; child was well nourished

Table 5 presents the results of the examination of the stools of four infants who were seriously ill.

Two observations show very high per cent. of total fat, in stools which were very different in appearance. One was large, green and watery; the other was approximately normal in appearance, but the daily amount was large. In both the per cent. of total fat is about the same as in a number of the normal stools, but the abnormal character is shown in the distribution of the fat. The soap is extremely low, much lower than is found in the stools of normal infants. The neutral fat and the fatty acids are both very high. The other two



cases show a very low percentage of total fat. The stools were green, watery and contained much mucus. Both these infants had a very low fat intake. One child was so ill that he nursed very little, and the other received milk with a very low fat content. In the stools of both these infants the soap per cent. of total fat is also very low.

A study of Tables 1 to 5 suggests an answer to two of the questions stated in the beginning of the paper, first, the normal per cent. of total fat and the distribution of the fat as soap, free fatty acids and neutral fat, and, second, the difference in fat content and distribution between normal and abnormal stools.

The fat per cent. of dried weight of the normal stools is high, usually over 30 per cent., and frequently reaches 50 or 60 per cent. with infants whose digestion is excellent. As the stools depart from the normal the per cent. of fat usually diminishes. When the fat per cent. is low it may be suspected that the stool contains an abnormal amount of mucus.

In the best stools the soap fat regularly exceeds in amount the other forms of fat and constitutes on the average nearly half the fat of the stool. Folin and Wentworth<sup>2</sup> have called attention to the possibility that during the process of drying a moist acid stool on a waterbath, a change may take place in the relation of the free fatty acids and the soaps, as a result of the volatilization of the lower acids, derived from sugar, such as lactic and acetic acids. On this account we have analyzed for comparative study a large quantity of composite stool obtained from about twelve breast fed infants. This composite stool appeared to be fairly representative of normal and nearly normal stools. It was found that although there is an increase of soap at the expense of the free fatty acids in the dried specimen, yet this difference is not great enough to alter our conclusion, founded on the analysis of the dried stools, namely, that in the best stools of breast fed infants the soap fat predominates over the other forms of fat. The results of the analyses of the composite stool, part of which was examined fresh and part of which was analyzed after drying on the waterbath, are as in Table 6.

2. Folin and Wentworth: Jour. Biol. Chem. 6:421, 1910.

In very acid stools which show even when dried a very low per cent. of soap, the difference between the analyses of the moist and dried material might be even greater than was found in the above-mentioned sample. In an alkaline composite stool, from children fed on cow's milk dilutions, no change in the distribution of soap and free fatty acids was caused by the drying process.

The increased difficulty of manipulation and the inconvenient necessity of making analyses immediately make it less desirable to work with the fresh stool. Hence, the use of dried material is more practicable, but the results must be interpreted with due consideration for the effect of drying on the relative proportions of free fatty acids and soap. From the foregoing investigation it would seem that, on the average, the soap value as determined in the dried stool should be decreased by about 10 per cent. of the total fat and the fatty acids

TABLE 6.  
*Analysis of Composite Stool.*

	Fat per Cent. of Dried Weight	Per Cent. of Total Fat as		
		Soap	Free Fatty Acids	Neutral Fat
Fresh sample.....	44.6	44.6	35.9	19.5
Dried sample.....	46.3	55.8	25.4	18.8

correspondingly increased in order to represent conditions in the fresh stool.

The neutral fat is normally less than 20 per cent. of the total fat. When the proportion of neutral fat is much higher than 20 per cent., an abnormal intestinal condition is indicated.

The variation in the fatty acids is striking. As the stools depart slightly from the normal the fatty acids are found to be increased at the expense of the soap fat; in those which are markedly abnormal the neutral fat is raised at the expense of the free fatty acids.

Abnormal stools are of two classes with respect to fat content: (1) Those containing much mucus with a low per cent., and (2) diarrheal stools, in which the fat per cent. is high, the result of poor absorption. Under abnormal conditions the per cent. of total fat which is soap is regularly low, while the neutral fat is high.

In thirty-two of the cases studied the per cent. of fat in the mother's milk was determined.<sup>3</sup> These figures are incorporated in Tables 1 to 5. No constant relation is shown between the per cent. of fat in the milk and the per cent. and distribution of fat in the stool. There is possibly a slightly higher fat per cent. in the stools of the infants who were receiving milk higher in fat, but the distribution of the fat of the stool does not seem to be influenced by the per cent. of fat in the milk taken.

In only 13 cases was the exact daily intake of fat known. These are shown in Table 7.

TABLE 7.

*Relation of Fat Intake to Fat Excretion and Distribution in the Stool.*

Case	Fat Intake, Gm. Daily	Fat per Cent. of Dried Weight	Per Cent. of Total Fat as		
			Soap	Free Fatty Acids	Neutral Fat
38. E. M. 2	42.5	8.8*	37.0	30.0	33.0
6. P. L. 3	41.9	41.0	63.8	25.8	10.6
4. P. L. 2	37.7	52.0	67.7	24.4	7.9
9. E. M. 1	37.5	32.7	56.9	21.7	21.4
33. J. F.	33.1	12.4*	51.6	24.5	23.9
2. P. L. 5	31.1	29.3	74.0	14.6	11.4
46. H. F.	25.2	53.7†	6.6	49.6	43.8
19. V. R.	19.8	30.8	45.2	No separation	
45. J. D.	15.3	57.1†	11.0	56.3	32.7
41. J. M.	10.6	14.4	34.2	34.3	31.5
20. N. K. 2	10.5	18.6	41.4	35.1	23.5
27. N. K. 1	7.0	27.3	27.5	55.8	16.7
47. C. V.	7.0	17.2	20.5	No separation	

\* Stool contained considerable mucus.

† Infant was very ill.

In the cases where the fat intake is high there is a somewhat higher per cent. of total fat in the stool and a higher proportion of the fat as soap than in the other cases with lower fat intake. The only marked exceptions to this are two instances in which a large proportion of the stool was mucus and two others in which the children were very ill.

3. A slight modification of the Babcock method was used to determine the fat per cent. of breast milk. It was found that when 17.6 c.c. of sulphuric acid, which is the rule in determining the fat of cow's milk, was used with breast milk the fat became charred and did not give a clear separation. If the amount of acid is reduced to 13 or 14 c.c. a clear separation of fat is obtained.

In general, the stools are more nearly normal when the fat intake is high. In other words, the normal condition in breast fed infants seems to be a high fat intake.

In eleven instances not only was a record of the exact daily fat intake obtained, but the stools were collected for definite periods, twenty-four or forty-eight hours. It is to be regretted that this number is so small, for there is need of more exact knowledge in regard to the absorption of fat by breast fed infants. Various investigators,

TABLE 8.

*Absorption of Fat by Normal Breast Fed Infants.*

Case	Age	Fat Intake, Gm. Daily	Excretion, Gm. Fat in Feces Daily	Per Cent. of Intake Absorbed	Condition of Child
38. E. M. 2	12 wk.	42.5	0.31	99.2	Gaining
33. J. F.	10 mo.	33.0	0.27	99.2	Healthy, gaining; stool green and full of mucus
2. P. L. 5	9 wk.	31.1*	1.61	94.9	Excellent condition; gaining
4. P. L. 2	3 wk.	37.7	1.95	94.8	Excellent condition; gaining
6. P. L. 3	3.5 wk.	41.9	2.26	94.5	Excellent condition; gaining
41. J. M.	4 mo.	10.6	0.62	94.2	Gaining very little; mother's milk abnormally low in fat and sugar
19. V. R.	3 mo.	19.8	1.22	93.9	Well nourished but not gaining at time of observation
17. D. T.	6 wk.	9.8	0.94	90.3	Gaining; previously marasmus
Average .....				95.1	

\* 4.3 grams fat from small amount of formula.

Michel, Keller, Rubner and Heubner, Freund, Uffelmann, Nobécourt and Merklin, report figures for fat absorption ranging from 92 to 99 per cent. This range is similar to that found in our cases. Table 8 presents the findings in eight observations on six normal infants and shows a range in absorption from 90.3 to 99.2 per cent. of the fat intake, and an average of 95.1 per cent.

In contrast to the foregoing, Table 9 shows how fat absorption is reduced under abnormal conditions. The three observations reported in this table were upon children acutely ill.



Attention should be called to the high intake in five of the cases in Table 8. With over 30 gm. of fat daily, all the children were gaining well and their subsequent progress was entirely satisfactory. An intake of 41.9 gm. of fat daily at the age of 3½ weeks, as in Observation 6, might be thought excessive, but this infant had stools entirely normal, and his condition was at the time, and continued to be, excellent.

The great loss of fat under abnormal intestinal conditions is shown by two of the observations in Table 9. One child, who was very sick with ileocolitis, but whose stools were not loose at the time, had a daily loss in the stool of 5.7 gm. of fat. Another child with severe diarrhea lost 12 gm. of fat daily in the stool.

TABLE 9.

*Absorption of Fat by Sick Breast Fed Infants.*

Case	Age	Fat Intake, Gm. Daily	Excretion, Gm. Fat in Feces Daily	Per Cent. of Intake Absorbed	Condition of Child
47. C. V.	5.5 mo.	7.0	1.48	79.0	Not gaining; eczema and diarrhea; mother's milk very low in fat
45. J. D.	8 mo.	15.7	5.71	62.6	Ileocolitis
46. H. F.	6 mo.	25.2	12.00	52.5	Acute diarrhea

In the literature of the fat metabolism of infants emphasis is frequently laid on the point that a high proportion of fat and particularly of soap fat in the stools is to be looked on as unfavorable. This belief is apparently based on the assumption that the stools of healthy breast fed infants, taken as a normal standard, contain a low per cent. of fat comparatively little of which is in the form of soap. The results obtained by us with so considerable a number of healthy breast fed infants are entirely at variance with this view.

## CONCLUSIONS.

1. The fat of the stools of normal breast fed infants, according to our observations, averaged 34.5 per cent. of the dried weight and frequently was as high as 50 per cent.

2. The soap fat in the best stools predominated over the other forms of fat, averaging 57.8 per cent. of the total fat, as determined on the dried stool. The average stool of the normal breast fed infants showed a soap fat of 43.1 per cent. of the total fat, as determined on the dried stool, which would correspond to over one-third of the total fat of the fresh stool.

3. The neutral fat in the best stools averaged 15.9 per cent. of the total fat; in the average stool the neutral fat was 20.2 per cent. of the total fat. The amount of neutral fat is not affected by the drying process.

4. No constant relation was shown between the per cent. of fat in the mother's milk and the per cent. of total fat and its distribution in the stool.

5. With a higher total intake of fat, the fat per cent. and the soap fat in the stool were somewhat increased.

6. A range of fat absorption from 90.3 to 99.2 per cent. of the intake was found in healthy breast fed infants.

We wish to acknowledge our thanks to the physicians of the New York Foundling Hospital and the Vanderbilt Clinic, and to the visiting physicians of the Babies' Hospital for their kind assistance in collecting stools.



## FAT METABOLISM OF INFANTS AND YOUNG CHILDREN.

### II. FAT IN THE STOOLS OF INFANTS FED ON MODIFICATIONS OF COW'S MILK.

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In a previous article of a series of studies on the fat metabolism of infants and young children we have reported the results of the examination of stools of breast fed infants. In the present paper are presented the results of the study of a large number of stools of infants fed on cow's milk or various modifications of the same, with or without carbohydrate additions. The digestion of the larger number of the infants was quite normal; some were suffering from disturbances of digestion of varying degrees of severity.

The questions here considered are practically the same as those discussed in the study of the stools of breast fed infants, namely:

1. What is the variation in the per cent. of total fat and the distribution of fat in the stools of infants fed on cow's milk, under different conditions of digestion?

2. How is the fat per cent. and the distribution of fat in the stool affected by the fat intake?

3. What per cent. of fat is retained under different conditions of digestion and how is the retention affected by the amount of fat intake?

The material examined consisted of 128 specimens of feces from seventy-seven infants whose ages ranged from 2 to 18 months. Some of the older infants who were taking whole milk are included because the amount of solid food they were receiving in addition was not sufficient to put them in the class with other children of their age on mixed diet. The exact food in each case is given in the tables. The milk formulas are expressed as per cent. of fat, carbohydrate and protein in the order stated. All additions to the milk—sugars, flours,



etc., have been included in the values as stated. Other additions to the diet—cereals, orange juice, olive or cod liver oil, etc.—are stated as such. The condition of the children with respect to digestion is considered in connection with the various tables.

The collected stools were dried on the waterbath to constant weight and ground to a powder. Analyses were made for total fat and its distribution as soap, free fatty acids and neutral fat. The findings numbered from 49 through 101 were obtained by the use of the method recently described by us,<sup>1</sup> and which was used to obtain the results in our previous paper on "Fat in the Stools of Breast Fed Infants."<sup>2</sup> The findings in the remaining cases, numbered from 102 through 176, were obtained by a modified Soxhlet extraction.<sup>3</sup> The separation of free fatty acids and neutral fat has not been included in this paper unless obtained by the titration method.

Since the soap content of the acid stools of breast fed infants is increased during the drying process, an investigation was made to determine whether a similar change occurs in the stools of children fed on cow's milk. Accordingly, a large composite specimen, representing normal or practically normal types of stools of artificially fed infants, was analyzed both in the moist state and after drying on the waterbath. It was found by this investigation that neither the total fat nor the distribution of fat was affected by drying. It is possible that acid diarrheal stools, like the acid stools of breast fed infants, might show a lower soap if analyzed moist than is present after drying.

The gross appearance of the stools is commonly used by pediatricists as their chief guide in infant feeding. Hence, the data presented have been classified according to the gross appearance of the stools and their water content.

Tables 1 to 9 are concerned with the fat per cent. of dried weight and the distribution of fat as soap, free fatty acids and neutral fat; Tables 10 to 18 consider the fat retention.

1. *Am. J. Dis. Child.* 17: 38 (Jan.) 1919.

2. *Am. J. Dis. Child.* 17: 241 (Apr.) 1919.

3. *Am. J. Dis. Child.* 3: 1 (Jan.) 1912.

*The Amount and Distribution of the Fat.*

Table 1 includes the findings on those stools which were hard, or dry and crumbly; that is, all the stools which might be regarded as

TABLE 1.

*Total Fat and Fat Distribution in Constipated Stools.*

No.	Case	Age, Months	Feeding			Intake of Fat, Gm. Daily	Analyses of Stools			
							Fat per Cent. of Dried Weight	Per Cent. of Total Fat as		
			Soap	Free Fatty Acids	Neutral Fat					
77	F. J.	15	Whole milk			30.0	35.1	89.2	7.9	2.9
67	M. R.	6	2.4	8.3	2.5	23.4	37.3	80.7	9.8	9.5
60	F. N. 1	11	2.6	11.7	2.9	26.2	44.5	80.4	10.0	9.6
			1 oz. orange juice							
71	P. D. 2	7	2.4	8.2	3.0	22.6	38.6	79.0	17.9	3.1
49	G. H.	7.5	4.1	4.9	2.2	48.3	48.1	78.0	9.5	12.5
			(top milk), 2 tsp. malt, & C. L. O.							
171	R. M. 3	9	2.5	6.8	2.5	31.5	31.4	77.4		
138	E. R. 1	4	1.4	5.0	1.2	13.2	25.9	77.2		
70	P. D. 1	7	2.4	8.2	3.0	22.9	36.3	76.4	18.7	4.9
128	A. R. 5	8	2.4	6.6	2.8	21.6	30.8	74.8	7.3	17.9
89	M. O. 2	5.5	1.5	5.0	2.7	11.7	36.7	74.5	17.9	7.6
			(evaporated milk)							
93	A. T.	3.5	1.5	5.0	2.7	9.5	15.7	74.1	18.6	7.3
			(evaporated milk)							
72	A. K.	5.5	2.8	5.0	2.6	22.1	46.1	72.9	18.0	9.1
75	M. H.	8	3.0	5.0	2.8	20.9	41.5	71.7	18.8	9.5
			1 tbsp. cereal							
65	P. D. 3	7.5	2.3	9.2	2.8	24.0	34.2	69.5	27.0	3.5
52	J. S.	15	Whole milk, C. L. O.,*			30.5*	39.5	69.1	19.9	11.0
			2 tbsp. cereal							
99	M. B. 2	13	Whole milk			17.0	34.3	68.1	21.4	10.5
61	E. C. 1	14	2.3	7.3	2.4	25.5	21.8	65.3	21.2	13.5
			2 tbsp. cereal, 1 prunes, 1 spinach							
90	W. D.	2.5	1.6	5.2	1.4	11.3	50.9	63.5	24.2	12.3
			(dried milk)							
69	M. L.	7	3.0	7.0	2.8	23.0	34.5	59.9	31.2	8.9
			2 tbsp. cereal							
Average . . . . .						22.9	36.0	73.8	17.6	9.0

\* Intake uncertain; cod liver oil indefinite.

constipated. Most of the infants could be classed as normal, healthy children, the rest, though under weight, were doing well at the time.

The fat per cent. of dried weight is quite uniform, averaging 36.0. There are only three values below 30, and only five above 40 per cent. The soap per cent. of total fat is very high, averaging 73.8. In thirteen instances the soap is over 70 per cent. of the fat in the stool. Of the other two constituents the fatty acids, averaging 17.6 per cent., exceed the neutral fat, which averages 9.0. In only two cases is this relationship reversed.

Table 2 presents the results of analyses of the stools of the type generally considered normal. They were formed or semiformed, smooth and homogeneous. They were not hard or dry and showed no signs of mucus. All the infants were in good condition as to digestion and were gaining weight.

The fat per cent. of dried weight in this group averages the same as that in Table 1, and the range is practically identical. The fat per cent. in the stool of E. M., 82.6 per cent., is the highest value that has ever come under our observation. This child was brought to the hospital suffering from forcible vomiting. She was taking 6 ounces of cream (the top 3 ounces from each of 2 quart bottles of milk) with 18 ounces of water and dextrimaltose. This formula was thus high in fat and carbohydrate and low in protein and salts. The child was also receiving two tablespoonfuls of milk of magnesia daily. After the collection of the stools for analysis, the food was changed and the vomiting ceased. In spite of the extremely high per cent. of fat in the stool of this child, the stool was normal in appearance, and it contained a high per cent. of soap. The large amount of magnesia taken may possibly have influenced the soap formation.

The distribution of fat shown in Table 2 is also almost identical with that in Table 1. The soap averages 72.8 per cent. of the total fat. There are three values lower than any found in Table 1, but Table 2 contains a proportionally larger number of high values. The averages for free fatty acids and neutral fat are practically the same in the two groups. Hence, as to total fat and distribution of fat, no distinction can be made between the stools of normal appearance and the hard, constipated type. The chief difference appears to be a matter of water content. This view is confirmed by a comparison of

TABLE 2.

*Total Fat and Fat Distribution in Stools of Normal Consistency.*

No.	Case	Age, Months	Feeding			Intake of Fat, Gm. Daily	Analyses of Stools			
							Fat per Cent. of Dried Weight	Per Cent. of Total Fat as		
			Fat	Carb.	Prot.			Soap	Free Fatty Acids	Neutral Fat
62	J. M.	7	2.2	6.7	2.0	24.4	20.6	89.3	6.1	4.6
81	R. B.	15	Whole milk, 4 tbsp. cereal			31.1	56.7	89.0	5.1	5.9
58	J. I.	12	2.4	8.7	2.6	28.6	22.4	85.8	6.2	8.0
			3 oz. orange juice							
87	B. I.	3.5	1.2	5.3	1.5	14.6	21.3	85.7	8.5	5.8
172	F. P. 1	3	1.4	6.7	1.4	14.7	45.4	85.2		
84	N. K. 3	2	2.4	4.8	2.1	15.1	15.7	81.0	10.9	8.1
73	P. S. 1	8	2.1	6.6	2.0	22.0	25.7	80.8	9.7	9.5
91	M. O. 1	5.5	1.5	5.0	2.7	11.5	39.0	80.0	12.1	7.9
			(evaporated milk)							
53	B. B.	3	3.0	7.0	1.8	27.8	50.2	79.2	14.6	6.2
			(top milk)							
76	P. S. 2	8	2.4	6.5	2.3	18.8	19.7	79.0	10.5	10.5
			1 oz. orange juice							
158	H. B.	13	Whole milk, 2 pc. bread, 2 tbsp. cereal			34.7	36.0	79.0	15.0	6.0
124	W. J. 1	5	2.2	2.9	1.7	23.3	38.7	78.9		
68	M. M.	11	2.1	8.0	2.5	23.3	21.4	77.6	9.9	12.5
			2 oz. potato, 1 tsp. butter							
55	J. D.	7	2.7	8.8	2.7	30.1	30.6	72.9	15.6	11.5
54	R. C.	8.5	2.5	6.0	2.4	32.8	28.2	72.8	18.2	9.0
			4 tbsp. cereal, 1 veg., 1 oz. orange juice							
57	M. S. 2	8	2.7	6.0	2.8	29.4	34.7	72.2	18.6	9.2
			1 tbsp. cereal, 1 tsp. veg., 1 oz. beef juice, 2 oz. orange juice							
168	W. J. 10	7	0.7	2.3	2.2	7.9	18.9	72.2		
96	R. L. 1	12	Whole milk			30.0	32.6	72.2	13.3	14.5
78	M. S. 1	4	1.7	5.0	1.8	16.7	42.9	72.0	23.0	5.0
140	R. M. 2	4.5	1.8	5.0	2.1	18.9	39.0	71.8		
50	B. P.	10	4.1	4.9	2.0	36.5	36.4	71.2	18.9	9.9
			(top milk)							
110	D. L.	8	2.2	7.3	2.0	24.1	36.0	70.1		
88	G. B.	3	1.5	6.5	3.0	12.7	33.3	68.5	19.6	11.9
			(evaporated milk)							



TABLE 2—*Concluded.*

No.	Case	Age, Months	Feeding			Intake of Fat, Gm. Daily	Analyses of Stools			
							Fat per Cent. of Dried Weight	Per Cent. of Total Fat as		
			Fat	Carb.	Prot.			Soap	Free Fatty Acids	Neutral Fat
105	R. S. 1	7	1.8	6.2	2.1	33.8	34.1	63.5		
			12 c.c. olive oil							
80	R. L. 3	12	Whole milk, 1 pc. bread, 1 tbsp. cereal			30.5	51.0	62.6	27.8	9.6
51	E. M. 1	2	5.0	5.0	0.9	36.0*	82.6	61.2	31.3	7.5
			(top milk)							
163	J. D.	5	1.8	5.0	2.1	18.9	46.5	59.1		
100	M. B. 1	13	Whole milk			17.0	38.1	56.4	24.7	18.9
122	V. D.	9	1.6	5.4	1.6	22.3	38.9	52.2		
			6 gm. olive oil							
79	R. L. 2	12.5	Whole milk, 1 pc. bread, 1 tbsp. cereal			30.5	48.6	43.7	42.7	13.6
117	W. H. 2	12	1.8	5.6	1.6	25.4	37.3			
			12 c.c. C. L. O.							
Average .....						24.0	36.2	72.8	16.5	9.4

\* Intake approximate; no sample of top milk available for examination.

the protein and ash content of the two types of stools. The average protein per cent. of dried weight of eight constipated stools was found to be 26.5, the average of eight normal stools 25.4. The average ash per cent. of dried weight of fifteen hard stools was 23.7, that of twenty normal stools was 24.1 per cent. of the dried weight. These averages were based on analyses made on the material considered in Tables 1 and 2. The most striking point brought out in Table 2 is that so many normal stools of healthy infants contain so high a proportion of their fat in the form of soap.

In Table 2 are presented the findings on a group of stools which were similar in appearance to the normal, but softer, for the most part not formed, but smooth and homogeneous and showing little or no mucus. None were included in this group that contained more than 75 gm. of water in the twenty-four hours' stool and none in which the dried weight was less than 14 per cent. of the moist weight.

All but three of the infants were in good condition and gaining weight, although several of them had previously suffered from digestive disturbances.

TABLE 3.

*Fat Content and Distribution in Stools Softer Than Normal.*

No.	Case	Age, Months	Feeding			Intake of Fat, Gm. Daily	Analyses of Stools			
							Fat per Cent. of Dried Weight	Per Cent. of Total Fat as		
								Soap	Free Fatty Acids	Neutral Fat
			Fat	Carb.	Prot.					
86	J. M.	4	1.6	5.2	1.4	13.4	34.5	80.4	11.0	8.6
			(dried milk)							
83	A. P.	6	1.8	5.0	2.2	15.1	18.7	75.9	14.3	9.8
63	F. N. 2	11	2.4	11.6	2.7	24.1	41.1	72.4	19.6	8.0
			1 oz. orange juice							
97	B. W. 1	15	Whole milk, half egg, 1 pc. zwieback			27.6	39.2	72.1	16.2	11.7
56	J. K. 1*	15	4.2	7.7	2.7	29.7	29.6	71.3	17.3	11.4
			(top milk)							
101	J. K. 2	16	Whole milk, 1 oz. beef juice, 1 tbsp. cereal, 1 fruit			16.5	12.8	68.7	19.6	11.7
94	E. W.	5	0.5	10.0	2.1	3.4	16.4	68.2	16.7	15.1
			(malting milk)							
74	E. C. 2	15	1.8	3.9	2.0	21.1	26.2	64.9	30.3	4.8
			2 tbsp. cereal, 1 prunes							
153	R. S. 3	8	2.4	8.2	2.3	27.7	25.6	62.9		
173	I. P. 1	4	1.1	10.5	1.6	20.2	39.0	58.1		
			8 gm. olive oil							
114	R. S. 2	8	2.4	8.2	2.3	27.7	29.0	57.9		
137	R. D. 1†	7.5	1.9	6.5	1.7	19.7	45.0	54.8		
103	W. J. 6	6	2.7	2.7	2.4	31.5	46.3	49.3		
145	R. M. 1	3.5	1.6	5.0	1.4	18.5	53.2	38.3		
66	P. S. 3	10	2.5	10.6	2.6	23.7	10.2	35.9	34.4	29.7
			15 c.c. orange juice							
64	L. R.‡	12	1.7	5.9	2.4	24.6	43.8	26.3	65.8	7.9
			12 c.c. cod liver oil							
Average . . . . .						21.5	31.9	59.8	24.5	11.9

\* Marked case of malnutrition.

† Gaining, but had a nasal infection with *K. L. bacillus*; subsequently very ill.

‡ Very delicate; rickets and marasmus.

Table 3 shows some marked variations from Tables 1 and 2. The total fat per cent. of dried weight is lower; the average being 31.9 per cent., and the range 10.2 to 53.2. The fat distribution shows an average for soap of 59.8 per cent., which is considerably lower than in the normal and the constipated stools; consequently, the fatty acids

TABLE 4.

*Fat Content and Distribution in Stools Which Were Not Homogeneous.*

No.	Case	Age, Months	Feeding			Intake of Fat, Gm. Daily	Analyses of Stools			
							Fat per Cent. of Dried Weight	Per Cent. of Total Fat as		
								Soap	Free Fatty Acids	Neutral Fat
			Fat	Carb.	Prot.					
59	L. H.*	9	2.6	6.0	1.8					
			2.3	5.2	2.0	19.8	27.5	77.8	17.5	4.7
98	A. K.	18	Whole milk, 4 tbsp. cereal, 1 tbsp. veg., 15 c.c. orange juice			26.4	41.4	76.2	14.4	9.4
92	L. B.*	3.5	1.7	9.7	1.4	12.5	45.2	67.3	17.8	14.9
82	P. L.*	4	1.8	5.2	1.6	15.7	26.1	64.7	27.7	7.6
			(dried milk)							
85	C. M.	2	1.6	5.0	1.7	14.4	33.4	64.4	23.7	11.9
155	D. W. 1†	5	1.8	5.0	1.6	17.2	48.8	55.2	13.1	31.7
154	W. H. 1*	12	1.8	5.6	1.5	17.4	14.8	54.2		
126	W. H. 4	14	2.4	6.2	2.3	35.8	38.6	50.5		
			1 tbsp. cereal, 12 c.c. C. L. O.							
104	C. R.	4	0.0	6.5	1.6	0.0	11.8	44.1		
174	B. B.*	4	1.4	6.7	1.4	14.7	54.7	41.2		
170	R. B. 2*	3	1.4	1.6	1.2	13.2	33.9	30.2		
119	W. H. 5	14	2.4	6.1	2.3	25.2	29.0	29.7		
			1 tbsp. cereal							
160	R. C. 1‡	5	0.9	5.4	2.0	6.7	18.8	23.4		
113	J. S. 1	7.5	1.2	5.0	3.2	26.6	13.1	16.8		
			14 c.c. olive oil							
169	R. C. 2‡	5	1.2	5.9	1.4	9.2	50.3	16.2		
166	A. A.*	7	1.4	6.9	2.0	11.4	35.6	1.6	21.1	77.3
Average .....						16.6	32.7	44.6	19.3	22.5

\* Child delicate; digestion not good.

† Child not in good condition; tetany; rickets; developed diarrhea.

‡ Child in bad condition; edema; idiot.

and neutral fat are higher, although they bear the same relation to each other as in the previous tables. The soap, however, still represents considerably more than half the fat of the stool.

Table 4 includes the findings on a number of stools which were not normal in appearance, but were not sufficiently loose to be classed as diarrheal. None were smooth and homogeneous, and all showed fat curds or mucus, or both, in considerable amount. The digestion of the children whose stools are considered in this table was not quite normal, and a large proportion of the infants subsequently developed diarrhea.

The total fat per cent. of dried weight of the stools in this group is not greatly different in average or in range from that of the previous tables. In this group the wide range may be explained by the fact that some of the stools contained considerable mucus, which diminishes the fat per cent., while others contained fat curds, which increase the per cent. of total fat. The most notable feature of this table is the extremely wide range in per cent. of soap fat. This is not surprising since the stools were classified together only because they contained curds and mucus and were not homogeneous. The soap fat forms on the average 44.6 per cent. of the total fat.

The group of stools, the analyses of which are shown in Table 5, were distinctly loose, containing from 70 to 100 gm. of water in the daily stool, which is more than twice the amount usually contained in normal stools. The infants observed were convalescent from digestive disturbance and were in fair condition.

The average proportion of the dried weight which is fat as shown in this table is similar to that of the two preceding tables, but the range is narrower. With the increase in the proportion of water in the stools a marked reduction of the soap per cent. of total fat is observed. In this group it averages 30.6 per cent. and there are only two values over 50 per cent.

The diarrheal stools are arranged in two groups according to the amount of water in the daily stools. Those which contained between 100 and 200 gm. of water daily are grouped together and are designated simply as diarrheal, and the findings are given in Table 6. The stools which contained more than 200 gm. of water daily are classed as severely diarrheal and the analyses are shown in Table 7. Most of



the children were very ill and the digestion of all was markedly abnormal.

In Table 6 the total fat, averaging 33.4 per cent. of the dried weight, shows no significant difference from that of the two preceding groups. Table 7, however, shows a marked increase in per cent. of total fat, averaging 40.7, the highest of all the groups. The per cent. of total fat which is soap shows a striking decrease in both these

TABLE 5.

*Fat Content and Distribution in Loose Stools.*

No.	Case	Age, Months	Feeding			Intake of Fat, Gm. Daily	Analyses of Stools			
							Fat per Cent. of Dried Weight	Per Cent. of Total Fat as		
								Soap	Free Fatty Acids	Neutral Fat
129	J. G. 4	8	2.8	6.5	2.5	32.3	38.1	54.0		
148	W. J. 3	6	1.6	4.7	2.1	18.3	31.7	50.2		
112	W. J. 5	7	2.3	2.6	2.3	27.0	47.8	40.8		
125	W. G. 2	7	2.1	4.9	2.6	22.5	29.1	35.1		
107	F. H. 11	13	2.4	6.8	2.5	29.9	18.1	32.6		
151	W. G. 1	7	1.7	4.8	2.4	17.9	20.2	30.8		
144	V. R. 2	3	1.6	5.0	1.4	18.7	24.0	30.4		
143	F. S.	6	1.7	6.0	2.1	18.7	36.1	28.3	16.6	55.1
108	W. G. 3	7.5	2.3	4.7	2.6	29.8	32.3	24.3		
			6 c.c. olive oil							
116	W. G. 4	7.5	1.7	4.5	2.2	25.5	41.2	17.6		
			8 c.c. olive oil							
115	F. H. 9	13	2.1	5.7	2.5	26.2	17.8	14.4		
159	D. F. 3	4	1.2	5.2	1.5	12.9	25.4	8.2		
Average .....						23.3	30.2	30.6	(16.6)	(55.1)

groups, averaging only 12.4 in Table 6 and 8.8 in Table 7. In Table 6 there is only one soap value over 27 per cent., while in the group of severe diarrhea, Table 7, there are only two instances with a soap per cent. above 10. Although the number of cases in which a separation of the neutral fat and the free fatty acids was obtained was small, the high per cent. of neutral fat in all shows that a much lessened degree of fat splitting is one of the accompaniments of diarrhea.

TABLE 6.

*Fat Content and Distribution in Diarrheal Stools.*

No.	Case	Age, Months	Feeding			Intake of Fat, Gm. Daily	Analyses of Stools			
							Fat per Cent. of Dried Weight	Per Cent. of Total Fat as		
								Soap	Free Fatty Acids	Neutral Fat
102	J. G. 3	7.5	2.2	9.0	2.2	25.4	27.3	47.9		
147	W. H. 3	14	2.4	6.1	2.3	18.4	22.3	27.4		
			1 tbsp. cereal							
146	J. G. 1	7	2.4	5.6	2.2	39.7	49.9	24.6		
			12 c.c. olive oil							
149	W. J. 2	5	1.7	6.3	1.7	18.1	36.2	21.1		
136	F. H. 2	10	2.7	3.8	2.2	33.6	29.5	20.3		
167	R. B. 3	3.5	1.1	3.2	1.2	9.2	34.1	20.8		
150	J. G. 2	7	2.4	5.6	2.2	30.7	33.8	18.7		
130	R. B. 1	3	1.4	5.5	1.2	13.2	16.6	16.3		
127	D. W. 2	5	1.8	5.8	1.6	22.2	48.9	15.3	23.2	61.5
			6 c.c. cod liver oil							
161	I. P. 2	4.5	1.4	12.0	1.8	24.4	25.4	15.1		
			8 c.c. olive oil							
162	D. F. 1	3.5	1.5	5.9	1.5	15.8	24.0	14.7		
165	P. V.	5.5	1.4	5.4	1.7	15.0	32.2	13.8		
139	J. D.	5	1.6	5.8	1.6	17.0	48.0	13.6	24.0	62.4
			3.4 c.c. cod liver oil							
134	D. F. 2	4	1.3	5.2	1.5	14.0	26.2	12.1		
135	F. H. 4	11	1.6	4.7	3.0	20.0	14.2	11.8		
142	W. J. 6	7	1.0	2.9	2.4	12.1	31.8	11.1		
175	I. G.	2.5	1.8	4.8	2.3	14.7	61.4	7.4	36.8	55.8
			(evaporated milk)							
118	A. H.	11	2.0	5.3	1.8	25.2	45.9	4.7		
152	M. R.	4	1.3	6.9	2.2	5.4	41.5	3.5	50.7	45.8
133	F. H. 10	13	1.6	6.7	2.5	20.6	16.4	1.6		
106	V. C. 3	5	1.8	2.8	2.1	30.2	54.2	0.0	17.5	82.5
			12 c.c. cod liver oil							
111	E. R. 2	10	1.8	6.3	2.2	27.1	31.6	0.0		
			4 tbsp. cereal,							
			12 c.c. cod liver oil							
123	E. R. 3	10.5	1.4	6.3	2.2	23.4	39.4	0.0		
			4 tbsp. cereal,							
			12 c.c. cod liver oil							
131	E. R. 1	10	2.0	8.7	2.7	16.8	44.1	0.0		
			4 tbsp. cereal							
164	B. S. 2	12	1.9	6.8	2.1	15.5	29.5	0.0		
			1 tbsp. cereal							
141	F. H. 3	11	0.0	6.0	3.5	0.0	3.9	0.0		
Average .....						19.5	33.4	12.4	30.4	61.6

TABLE 7.

*Fat Content and Distribution in Severely Diarrheal Stools.*

No.	Case	Age, Months	Feeding			Intake of Fat, Gm. Daily	Analyses of Stools			
							Fat per Cent. of Dried Weight	Per Cent. of Total Fat as		
			Fat	Carb.	Prot.			Soap	Free Fatty Acids	Neutral Fat
157	E. R. 2	4.5	1.3	5.9	1.2	11.8	30.2	22.8		
132	F. H. 1	10	2.6	4.5	2.4	32.4	34.7	17.0		
95	J. B.	4	1.3	3.5	1.6	6.4	46.1	9.8	43.2	37.0
			(evaporated milk)							
156	W. S. 1	4	1.6	5.8	1.5	17.0	36.6	8.6		
109	S. J.	9	2.7	5.3	2.5	29.1	57.5	8.9	36.3	54.8
			(evaporated milk)							
121	V. C. 2	5	1.8	2.9	2.1	23.6	49.5	3.3	30.3	46.4
			6 c.c. cod liver oil							
120	V. C. 1	5	1.8	5.6	2.0	23.6	43.6	0.0	32.7	67.3
			6 c.c. cod liver oil							
176	B. S. 3	13	1.8	5.9	1.9	11.6	27.0	0.0		
			one-half tbsp. cereal							
Average .....						19.4	40.7	8.8	38.1	56.4

TABLE 8.

*Summary of Averages of Tables 1 to 7.*

Table	Type of Stools	Number of Observations	Intake of Fat, Gm. Daily	Analyses of Stools			
				Fat per Cent. of Dried Weight	Per Cent. of Total Fat as		
					Soap	Free Fatty Acids	Neutral Fat
1	Constipated .....	19	22.9	36.0	73.8	17.6	9.0
2	Normal .....	31	24.0	36.2	72.8	16.5	9.4
3	Softer than normal .....	16	21.5	31.9	59.8	24.5	11.9
4	Not homogeneous .....	16	16.6	32.7	44.6	19.3	22.5
5	Loose .....	12	23.3	30.2	30.6	16.6*	55.1*
6	Diarrheal .....	26	19.5	33.4	12.4	30.4	61.6
7	Severely diarrheal .....	8	19.4	40.7	8.8	38.1	56.4

\* Only one case.

For comparison, the averages of the seven previous tables have been brought together in Table 8.

This table gives an answer to the first question stated at the beginning of this article, namely, What is the variation under different conditions of digestion in the per cent. of total fat and the distribution of fat in the stools of artificially fed infants?

The average values for the fat per cent. of dried weight in the various groups range from 30 to 40 per cent. The constipated stools have the same average per cent. as the normal. The fat average is highest in the group of severe diarrhea. In the groups of stools intermediate between the normal and those of severe diarrhea the total fat per cent. of dried weight is lower than in the normal. This may be due to the presence in these types of stools of more or less mucus. Undoubtedly the stools in severe diarrhea also contain much mucus, but in this condition the absorption of fat is so very poor that there is an extremely large amount of fat in the stool and a consequently high per cent. of the dried weight in spite of the presence of much mucus.

The distribution of fat shows wide variation according to the type of the stool. The stools which were normal and nearly normal in appearance show very high average values for soap. It is only when the stools show in their gross appearance evidence of digestive disturbance that the average values for soap drop below 50 per cent. of the total fat. In diarrheal stools the soap forms a very small proportion of the total fat. The fatty acids form the next larger proportion of the total fat in good stools, the lower neutral fat indicating excellent splitting of the ingested fat. In the diarrheal stool the proportion of neutral fat is greatly increased, forming on the average more than half the total fat.

Table 9 shows for comparison the average analyses of the stools of nursing infants and of infants taking cow's milk modifications. It gives average analyses of the normal stools and also the averages of the analyses of all the stools not diarrheal. All the children, the analyses of whose stools are included in the second average, were in fairly good condition as to digestion, most of them in excellent condition.



The normal stools of nursing infants show a higher average fat per cent. of dried weight, 42.1, than do the stools of infants fed on modifications of cow's milk, 36.2. But in the average which includes all the various types of stools exclusive of diarrheal, the fat per cent. of dried weight is exactly the same with both cow's milk and breast milk feeding. The soap per cent. of total fat is much higher in the stools of infants taking cow's milk than in those of infants taking breast milk, both in the case of normal stools and that of various types whose analyses were averaged together. This difference would have been even greater if the determinations had been made on the

TABLE 9.

*Comparison of Analyses of Stools of Infants Taking Cow's Milk and Those Taking Breast Milk.*

Food	Type of Stool	Fat per Cent. of Dried Weight	Per Cent. of Total Fat as		
			Soap	Free Fatty Acids	Neutral Fat
Cow's milk .....	Normal (Table 2) .....	36.2	72.8	16.5	9.4
Breast milk .....	Normal (Table 1)* .....	42.1	57.8	26.3	15.9
Cow's milk .....	Average† (Tables 1 to 5) ..	34.1	60.5	18.6‡	12.1‡
Breast milk .....	Average† (Tables 1 to 3)* ..	34.5	43.1	36.7	20.2

\* Preceding paper. (Fat in the Stools of Breast Fed Infants.)

† Average of analyses of all types of stools except diarrheal. All the children were in fairly good condition as to digestion, most of them in excellent condition.

‡ Since the separation of free fatty acids and neutral fat was not obtained on all the stools, the averages of soap, free fatty acids and neutral fat do not total 100 per cent.

moist stools, since a lower soap value is found in the stools of nursing infants when examined moist than is found in the dried stool, while no such difference is found in the stools of infants taking cow's milk.

The neutral fat is distinctly higher in the stools of breast fed infants. The values for neutral fat need no correction for purposes of comparison, since the amount of neutral fat is not changed by the process of drying. From the foregoing facts the inference would seem to be warranted that in artificial feeding the aim should be to obtain a stool with a high proportion of soap fat rather than one in which the soap is lower, as it is in the stools of nursing infants.

The amount of daily fat intake has been incorporated in Tables 1 to 7. A study of these figures fails to reveal any definite relation between the fat intake and the per cent. of total fat or its distribution in the stool. The intake varied in the cases studied from 0 to 48.3 gm., the average intake for the various groups ranging from 16 to 24 gm. A large number of the cases with high fat intake appear in Tables 1 and 2. The per cent. of fat in the formula seems to bear no definite relation either to the per cent. of fat in the stool or its distribution.

### *Retention of Fat.*

In about three-fourths of the cases considered in the preceding tables, the stools were collected for a definite period, so that the actual and percentage retention of the fat intake was obtained. These values are also arranged according to the gross appearance and water content of the stool.

The first group, Table 10, includes the findings on those cases in which the stools were constipated.

TABLE 10.

#### *Retention of Fat When Stools Were Constipated.*

No.	Case	Intake of Fat, Gm. Daily	Fat in Stool, Gm. Daily	Fat Retained, Gm. Daily	Per Cent. of Intake Retained
49	G. H.	48.3	2.98	45.3	93.8
171	R. M. 3	31.5	3.12	28.4	90.2
77	F. J.	30.0	1.75	28.3	94.2
60	F. N. 1	26.2	4.12	22.1	84.3
61	E. C. 1	25.5	1.71	23.8	93.3
65	P. D. 3	24.0	2.95	21.1	87.7
67	M. R.	23.4	2.79	20.6	88.0
70	P. D. 1	22.9	2.21	20.7	90.3
71	P. D. 2	22.6	3.34	19.3	85.2
72	A. K.	22.1	3.04	19.1	86.2
128	A. R. 5	21.6	2.12	19.5	90.1
	Average.....	27.1	2.74	24.4	89.9

The per cent. of the fat intake retained ranges from 84 to 94, with an average of nearly 90. The largest intake in all the cases studied was that of G. H. (No. 49). This child retained 93.8 per

cent. of the unusually large intake of 48.3 gm., having an actual retention of 45.3 gm. In the case of F. N. (No. 60), who had the largest loss of fat in the stool, the food contained an unusually high proportion of carbohydrate.

Table 11 presents the figures for fat retention in the cases in which the stools were of normal consistency.

The range in retention is from 85.8 to 97.4 per cent. of the fat intake, the average being 91.3. Three cases (Nos. 81, 96, 158) show

TABLE 11.  
*Retention of Fat When the Stools Were Normal in Appearance.*

No.	Case	Intake of Fat, Gm. Daily	Fat in Stool, Gm. Daily	Fat Retained, Gm. Daily	Per Cent. of Intake Retained
50	B. P.	36.5	2.91	33.6	92.1
158	H. B.	34.7	4.93	29.8	85.8
105	R. S. 1	33.8	2.37	31.4	93.1
54	R. C.	32.8	2.70	30.1	91.8
81	R. B.	31.0	3.91	27.1	87.4
55	J. D.	30.1	1.35	28.8	95.6
96	R. L. 1	30.0	4.07	25.9	86.4
57	M. S. 2	29.4	3.36	26.0	88.6
58	J. I.	28.6	1.30	27.3	95.4
117	W. H. 2	25.4	2.24	23.2	91.2
62	J. M.	24.4	1.14	23.3	95.3
110	D. L.	24.1	3.41	20.7	86.0
68	M. M.	23.3	1.24	22.1	94.7
122	V. D.	22.3	2.76	19.5	87.6
73	P. S. 1	22.0	0.73	21.3	96.7
140	R. M. 2	18.9	2.59	16.3	86.2
76	P. S. 2	18.8	0.62	18.2	96.7
87	B. L.	14.6	0.38	14.2	97.4
	Average.....	26.7	2.33	24.4	91.3

a high loss of fat, comparable with that of No. 60 in the preceding table. These three infants were all fed on whole milk, two of them having cereal additions. A comparison of Tables 10 and 11 shows that there is no significant difference in the average daily loss of fat whether the stools are hard and constipated or of normal consistency. In Table 11, representing normal stools, there are seven instances in which there is a smaller daily fat loss than the lowest reported in Table 10 in which the stools considered were constipated.

In Table 12 is shown the retention of fat when the stools were nearly normal but contained a larger proportion of water.

In this table are seen several values for percentage retention of fat lower than any found in the two preceding tables, and the average, 87.9 per cent., is somewhat lower. A very high fat excretion is shown in three instances (Nos. 63, 64, 103); in only one of them was the intake high. Three others show very small fat excretion, but in two of them the intake was also low.

TABLE 12.

*Retention of Fat When the Stools Were Softer Than Normal.*

No.	Case	Intake of Fat, Gm. Daily	Fat in Stool, Gm. Daily	Fat Retained, Gm. Daily	Per Cent. of Intake Retained
103	W. J. 4	31.5	6.75	24.8	78.6
56	J. K. 1	29.7	2.48	27.2	91.7
114	R. S. 2	27.7	2.49	25.2	91.0
153	R. S. 3	27.7	3.22	24.5	88.5
97	B. W. 1	27.6	2.54	25.1	90.8
64	L. R.	24.6	4.60	20.0	81.2
63	F. N. 2	24.1	5.09	19.0	78.8
66	P. S. 3	23.7	0.85	22.9	96.3
74	E. C. 2	21.1	2.71	18.4	87.2
173	I. P. 1	20.2	2.10	18.1	89.6
145	R. M. 1	18.5	2.81	15.7	84.8
101	J. K. 2	16.5	0.66	15.8	96.0
83	A. P.	15.1	0.65	14.6	95.7
86	J. M.	13.4	2.08	11.3	84.6
	Average.....	23.0	2.79	20.2	87.9
94	E. W.	3.4	1.09	2.3	68.3

In calculating the averages in this and following tables we have not included any cases in which the intake was less than 10 gm. It is noteworthy that when the intake was below this amount the per cent. of retention in our cases was always very low.

Table 13 shows the fat retention in a group of cases which are classed together because the stools were not smooth or homogeneous, but contained fat curds or mucus, or both.

In this group the range of retention is wider than in the preceding, being from 73.2 to 96.5 per cent. of the intake, with an average of



TABLE 13.

*Retention of Fat When the Stools Were Not Homogeneous.*

No.	Case	Intake of Fat, Gm. Daily	Fat in Stool, Gm. Daily	Fat Retained Gm. Daily	Per Cent. of Intake Retained
126	W. H. 4	35.8	3.35	32.5	90.7
113	J. S. 1	26.6	1.53	25.1	94.3
98	A. K.	26.4	1.61	24.8	93.9
119	W. H. 5	25.2	2.64	22.6	89.6
59	L. H.	19.8	0.69	19.1	96.5
154	W. H. 1	17.4	0.91	16.5	94.8
155	D. W. 1	17.2	3.73	13.5	78.3
82	P. L.	15.7	1.73	14.0	89.0
85	C. M.	14.4	1.64	12.8	88.8
170	R. B. 2	13.2	3.52	9.7	73.2
92	L. B.	12.5	2.06	10.4	83.5
166	A. A.	11.4	2.46	8.9	78.2
	Average.....	19.6	2.16	17.5	89.1
169	R. C. 2	9.2	3.16	6.0	65.6
160	R. C. 1	6.7	1.29	5.4	80.7
104	C. R.	0.0	0.33	....	Neg.

TABLE 14.

*Retention of Fat When the Stools Were Loose.*

No.	Case	Intake of Fat, Gm. Daily	Fat in Stool, Gm. Daily	Fat Retained, Gm. Daily	Per Cent. of Intake Retained
129	J. G. 4	32.3	4.48	27.8	86.2
107	F. H. 11	29.9	2.15	27.8	92.8
108	W. G. 3	29.8	4.32	25.5	85.5
112	W. J. 5	27.0	7.57	19.4	71.9
115	F. H. 9	26.2	2.29	23.9	91.2
116	W. G. 4	25.5	6.10	19.4	76.1
125	W. G. 2	22.5	2.85	19.7	87.3
144	V. R. 2	18.7	1.88	16.8	90.0
143	F. S.	18.7	4.20	14.5	77.6
148	W. J. 3	18.3	4.01	14.3	78.1
151	W. G. 1	17.9	2.04	15.9	88.6
159	D. F. 3	12.9	2.93	10.0	77.3
	Average.....	23.3	3.74	19.6	83.9

89.1. There is here no essential variation from the normal either in average daily fat excretion or average per cent. of retention. The fat loss is slightly lower than that shown in the normal group; but this may be due to the lower intake.

The next group, presented in Table 14, shows the fat retention when the stools were loose.

TABLE 15.  
*Retention of Fat with Diarrhea.*

No.	Case	Intake of Fat, Gm. Daily	Fat in Stool, Gm. Daily	Fat Retained, Gm. Daily	Per Cent. of Intake Retained
146	J. G. 1	39.7	7.83	31.9	80.3
136	F. H. 2	33.6	4.61	29.0	86.3
150	J. G. 2	30.7	4.77	25.9	84.5
106	V. C. 3	30.2	6.73	23.5	77.7
111	E. R. 2	27.1	3.76	23.3	86.2
102	J. G. 3	25.4	3.98	21.4	84.3
118	A. H.	25.2	4.23	21.0	83.2
161	I. P. 2	24.4	2.99	21.4	87.8
123	E. R. 3	23.4	4.97	18.4	78.7
127	D. W. 2	22.2	5.38	16.8	75.7
133	F. H. 10	20.6	2.06	18.5	89.9
135	F. H. 4	20.0	1.77	18.2	91.1
147	W. H. 3	18.4	2.10	16.3	88.6
139	J. D.	17.0	6.01	11.0	64.7
131	E. R. 1	16.8	3.35	13.5	80.1
162	D. F. 1	15.8	3.07	12.7	80.5
164	B. S. 2	15.5	3.07	12.4	80.2
165	P. V.	15.0	4.42	10.6	70.6
175	I. G.	14.7	11.62	3.1	20.8
134	D. F. 2	14.0	3.23	10.8	77.0
130	R. B. 1	13.2	2.70	10.5	79.8
142	W. J. 6	12.1	5.77	6.3	52.3
	Average.....	21.6	4.47	17.1	79.3
152	M. R.	5.4	3.61	1.8	33.3
141	F. H. 3	0.0	0.43	....	Neg.

The average per cent. of the intake retained in this group is 83.9, the range being from 71.9 to 92.8. The fat excretion several times reaches a high figure and the average is distinctly higher than in any of the preceding groups. None of the infants, however, were in a serious condition.

In Table 15 are grouped the cases in which the daily stools contained between 100 and 200 gm. of water; that is, cases which would be classed as moderate diarrhea.

A decided increase in the fat loss and corresponding decrease in fat retention appears. The fat retention averages 79.3. The average daily fat excretion is somewhat higher than in the preceding groups, ranging from 1.77 to 11.62 gm. The child, I. G. (No. 175), who lost 11.62 gm. of fat in his daily stool, was fed on evaporated milk cooked with a considerable addition of starch. His stools were very large and showed fermentation; the appearance, however, was not that of

TABLE 16.  
*Retention of Fat with Severe Diarrhea.*

No.	Case	Intake of Fat, Gm. Daily	Fat in Stool, Gm. Daily	Fat Retained, Gm. Daily	Per Cent. of Intake Retained
132	F. H. 1	32.4	6.96	25.4	78.6
109	S. J.	29.1	14.54	14.6	50.0
120	V. C. 1	23.6	5.77	17.8	75.7
121	V. C. 2	23.6	10.38	13.2	56.1
156	W. S. 1	17.0	8.61	8.4	49.4
157	E. R. 2	11.8	5.56	6.2	53.0
176	B. S. 3	11.6	10.08	1.5	12.9
	Average.....	21.3	8.84	12.5	58.4
95	J. B.	6.4	12.58*		

\* Child recently changed from higher fat intake.

a diarrheal stool but the water content, 172 gm., and the total solids, 18.93 gm., were very high.

Table 16 shows the fat retention in the cases of severe diarrhea; that is, those in which the stools contained over 200 gm. of water daily.

This table illustrates strikingly the very great loss of fat which occurs in severe diarrheas. The smallest daily loss is over 5 gm., and the average is 8.8 gm. The retention ranges from 12.9 to 78.6 per cent. of the intake, the average being 58.4 per cent. The child, S. J. (No. 109), showed a condition similar to that of I. G. (No. 175) of the preceding table. The fat lost in the stools in this case was the greatest

amount found in our entire series, being 14.54 gm. daily. The food in this case was similar to that of I. G., that is, evaporated milk with cooked starch.

The significance of the findings reported in Tables 10 to 16 is brought out more clearly if the averages of the different tables are considered together. These are given in Table 17.

We have already stated that the groups with hard and with normal stools show no essential difference in fat retention. These groups, with the highest average intake, show the highest average per cent. of retention. In fact, in the first four groups the differences in the amount of fat excreted and the per cent. of fat retained are insignificant. It is not until the stools become loose that the fat excretion

TABLE 17.

*Summary of Average Fat Retention.*

Table	Type of Stools	No. of Observations	Intake of Fat, Gm. Daily	Fat in Stool, Gm. Daily	Fat Retained, Gm. Daily	Per Cent. of Intake Retained
10	Constipated.....	11	27.1	2.74	24.4	89.9
11	Normal.....	18	26.7	2.33	24.4	91.3
12	Softer than normal.....	14	23.0	2.79	20.2	87.9
13	Not homogeneous.....	12	19.6	2.16	17.5	89.1
14	Loose.....	12	23.3	3.74	19.6	83.9
15	Diarrheal.....	22	21.6	4.47	17.1	79.3
16	Severely diarrheal.....	7	21.3	8.84	12.5	58.5

becomes considerably increased and the retention correspondingly diminished. A comparative study of the last three groups shows strikingly the effect of an increase in the amount of water in the stools. The average daily fat excretion in loose stools is at least a gram more than that of any of the four preceding groups. In the diarrheal stools the average daily fat loss is much greater, nearly twice that shown in normal stools, and in severe diarrhea it is nearly four times the normal.

Table 18 shows the fat retention of infants fed on cow's milk in comparison with that of breast fed infants. In order to obtain values comparable with those obtained for the breast fed infants in good condition, the figures in Tables 10 through 14 have been averaged.



This gives the average fat loss and retention of all the infants fed on cow's milk modifications whose stools were not diarrheal.

The fat retention of the infants taking cow's milk averages 88.6 per cent. of the intake; of those taking breast milk it averages 95.1 per cent. of the intake. The daily loss of fat by infants fed on cow's milk is over twice as great as that lost by those taking breast milk, even though the actual amount of intake is somewhat less.

TABLE 18.

*Comparison of Fat Retention of Infants Taking Cow's Milk and Those Taking Breast Milk.*

Food	Type of Stools	Intake of Fat, Gm. Daily	Fat in Stool, Gm. Daily	Fat Retained, Gm. Daily	Per Cent. of Intake Retained
Cow's milk.....	Average* (Tables 10 to 14)	24.2	2.72	21.4	88.6
Breast milk.....	Average* (Table 8)†.....	28.3	1.15	27.2	95.1

\* Average of findings when the stools were of all types except diarrhea. All the children were in fairly good condition as to digestion, most of them in excellent condition.

† Preceding paper. (Fat in the Stools of Breast Fed Infants.)

#### SUMMARY.

1. The material presented in this article comprises the results of analysis of 128 stools of seventy-seven infants whose ages ranged from 2 to 18 months, fed on modifications of cow's milk.

2. The average fat per cent. of the dried weight in normal stools was 36.2. The hard, constipated stools showed no variation from this figure. In the stools not quite normal in appearance the average fat per cent. was slightly lower. In severe diarrhea the fat per cent. of dried weight was much higher, reaching an average of 40.7 per cent.

3. The soap per cent. of total fat was very high in both normal and constipated stools, averaging, respectively, 72.8 and 73.8 per cent. As the stools became less normal in appearance the soap fat diminished rapidly and averaged in the loose stools only 30.6 per cent. of the total fat, in the diarrheal stools 12.4 per cent., and in those of severe diarrhea only 8.8 per cent. of the total fat.

4. The neutral fat was less than 10 per cent. of the total fat in normal and constipated stools. It increased as the soap fat diminished and in diarrheal conditions made up about 60 per cent. of the total fat in the stool.

5. The free fatty acids constituted about 17 per cent. of the total fat of normal and of constipated stools. It was increased somewhat as the stools became less like the normal and in the diarrheal stools was over 30 per cent. of the total fat of the stool.

6. No definite relationship was shown between the daily fat intake and the per cent. of fat or the distribution of fat in the stool.

7. The average per cent. of the fat retained with normal stools was 91.3 per cent. of the intake. The retention was but little lower when the stools were somewhat harder or softer than normal, or were not homogeneous, or contained more or less mucus without being distinctly watery. As the water in the stools increased, the per cent. of retention dropped markedly, reaching in severe diarrhea 58.4 per cent. of the intake.

8. There was no striking relation between the fat intake and the per cent. of the intake retained, except when the intake was abnormally low.



## FAT METABOLISM OF INFANTS AND YOUNG CHILDREN.

### III. FAT IN THE STOOLS OF CHILDREN ON A MIXED DIET.

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Babies' Hospital.)*

This study of fat metabolism presents observations on children whose diet contained a large proportion of solid food. It gives the findings as to fat percentage and distribution in the stools of a number of children receiving a mixed diet and the fat retention of these children.

The material examined consisted of 134 collections of the feces of sixty-two children from one to ten years of age. Most of the children were between eighteen months and five years of age. The stools were dried and analyzed according to a procedure we have already described.<sup>1</sup> Only four specimens (Nos. 235, 236, 332, 333) were analyzed by the modified Soxhlet method. The remainder were analyzed according to the modification of the Roesse-Gottlieb method published by us. The values for fat intake in this paper, although carefully estimated, were not usually obtained by analysis and therefore must be considered as being approximate. When the children were not under our direct supervision, exact directions were given for collecting the stools and a record of the food was obtained for twenty-four hours preceding the collection of feces, as well as for the twenty-four or forty-eight hours period of collection. The fat was determined by the Babcock method in samples of the milk consumed by the children, and in the other food the fat was estimated. When practicable, the fat content of the solid part of the food was determined by taking an aliquot part, which was dried, ground and analyzed by the Roesse-Gottlieb method.

1. Am. J. Dis. Child. 17: 6 (June) 1919.



The larger number of the children whose stools were examined were in normal condition, both as to general health and digestion. Six of the children were rachitic; eleven were suffering from chronic intestinal indigestion, several being examples of intestinal infantilism.

The first part of the paper contains the observations made on healthy children whose digestion was nearly or quite normal; the second part contains the observations on the abnormal cases.

Tables 1, 2, 3, 4 and 5 give the fat per cent. and distribution of fat in the stools of the normal children, and Tables 6, 7, 8, 9 and 10 the retention of fat in most of these cases. In a few instances the stools were not collected for an exactly known period, and consequently the values for retention could not be used.

### *1. Fat Per Cent. and Distribution of Fat in the Stools of Normal Children.*

The findings on the stools of several children fed on milk alone or milk with bread and cereal are presented in Table 1. All these children had previously taken a more general diet. Four of the stools were constipated (Nos. 226, 227, 228, 229); the others were of normal consistency.

The average fat per cent. of dried weight, 30.7, shown in Table 1, is lower than that found in the stools of the infants on modifications of cow's milk. The soap per cent. of total fat is, on the whole, high, being highest in three of the stools which were constipated. The average soap per cent. for the group, 60.9, is lower than that found by us in normal and constipated stools of infants. Fatty acids are higher than neutral fat in every case but one, which was also true in similar stools of the infants. As far as the analyses of their stools are concerned, this group occupies a place between the infants on milk modifications and the older children on a mixed diet.

The greater number of the observations reported in this paper were made on the stools of children on a diet consisting of considerable amounts of solid food and usually a pint or more of milk. The findings have been arranged in three groups according to the gross appearance of the stools; those which were constipated (Table 2), those of normal consistency (Table 3) and those not normal (Table 4).

In the food column in these tables we have enumerated the articles which contained any considerable amount of fat, such as milk, butter, eggs, cod liver oil, etc. The remainder of the diet consisted of carbohydrate foods, vegetables and fruit.

In Table 2, which reports the findings on the constipated stools, the average for the daily fat intake is 40.9 gm. The fat per cent. of dried

TABLE 1.

*Fat Per Cent. and Distribution of Fat in Stools of Children on a Diet of Milk or Milk with Bread and Cereal.*

No.	Case	Age	Diet	Intake of Fat, Grams Daily	Analyses of Stools			
					Fat per Cent. of Dried Weight	Per Cent. of Total Fat as		
						Soap	Free Fatty Acids	Neutral Fat
226	C. A. 2	2 yr. 3 mo.	35 oz. milk, barley gruel.....	35.0	33.6	79.7	10.9	9.4
228	E. D. 2	2 yr. 6 mo.	40 oz. milk, 6 tbsp. cereal, 4 pc. bread.....	42.2	27.1	75.1	14.1	10.8
227	E. D. 1	2 yr. 6 mo.	35 oz. milk.....	35.0	39.5	71.2	21.2	7.6
225	C. A. 1	2 yr. 3 mo.	35 oz. milk.....	35.0	45.2	69.0	22.8	8.2
230	P. M.	2 yr. 4 mo.	20 oz. milk, 6 tbsp. cereal.....	21.1	26.3	64.4	30.7	4.9
231	V. G.	1 yr. 4 mo.	26 oz. skimmed milk, 5 tbsp. cereal, 1 pc. bread.....	15.0	38.8	61.5	26.9	11.6
233	F. W. 8	3 yr. 7 mo.	30 oz. milk, 4 tbsp. cereal.....	31.0	34.2	58.7	30.4	10.9
232	J. E.	1 yr. 11 mo.	27 oz. milk, 2 tbsp. cereal, 1 tbsp. vegetable, 2 pc. bread...	28.0	20.0	57.2	19.1	23.7
229	M. S.	10 yr.	32 oz. milk.....	32.0	24.7	40.7	37.2	22.1
234	A. L.	3 yr.	20 oz. milk, 6 tbsp. cereal, 2 pc. bread.....	21.8	17.7	31.0	38.1	30.9
Average .....				29.6	30.7	60.9	25.1	14.0

weight of the stools averages 20.1, which is much lower than was found when the diet was mainly milk. The average soap per cent. of total fat, 47.9, is also lower than that seen in Table 1, but the soap predominates over fatty acids and neutral fat in all but the last five instances. The average values for neutral fat and fatty acids are nearly the same; sometimes the fatty acids, sometimes the neutral fat show the larger percentage.

TABLE 2.

*Fat Per Cent. and Distribution in Stools of Normal Children on a Mixed Diet. Stools Constipated.*

No.	Case	Age	Diet	Intake of Fat, Grams Daily	Analyses of Stools				
					Fat per Cent. of Dried Weight	Per Cent. of Total Fat as			
						Soap	Free Fatty Acids	Neutral Fat	
280	B. W. 3	1 yr. 9 mo.	40 oz. milk, egg, C.L.O., etc...	55.5	33.7	75.2	11.4	13.4	
318	D. L.	1 yr. 2 mo.	25 oz. milk, egg, etc.....	33.6	33.6	65.4	23.7	10.9	
268	O. W. 2	2 yr. 7 mo.	32 oz. milk, egg, meat, etc...	38.5	29.8	64.6	16.5	18.9	
308	H. F. 5	1 yr. 1 mo.	24 oz. fat-free milk, egg, butter, etc.....	35.2	16.3	61.3	18.8	19.9	
326	E. A. 2	7 yr. 5 mo.	19 oz. skimmed milk, meat, butter, C.L.O., etc.....	34.0	31.1	56.4	27.8	15.8	
320	J. O. 2	3 yr.	32 oz. milk, egg, meat, etc...	38.3	17.0	56.2	18.2	25.6	
283	B. W. 6	2 yr.	32 oz. milk, egg, etc.....	42.1	24.0	55.5	19.5	25.0	
305	H. F. 1	1 yr.	32 oz. milk, egg, etc.....	34.9	21.5	53.2	22.7	24.1	
269	O. W. 5	2 yr. 8 mo.	32 oz. milk, egg, meat, C.L.O., etc.....	53.5	21.8	53.0	28.2	18.8	
307	H. F. 3	1 yr.	24 oz. skimmed milk, butter, egg, etc.....	36.6	18.9	51.2	29.0	19.8	
321	F. S.	1 yr. 6 mo.	40 oz. milk, egg, nut butter, meat, etc.....	61.0	23.4	51.2	24.1	24.7	
270	A. W. 1	4 yr.	32 oz. milk, egg, meat, etc...	38.4	13.4	47.1	29.7	23.2	
284	B. W. 12	2 yr. 3 mo.	32 oz. milk, egg, etc.....	40.5	17.8	46.3	26.9	26.8	
306	H. F. 2	1 yr.	26 oz. milk, egg, butter, etc...	36.5	19.0	45.5	25.6	28.9	
310	R. K. 1	1 yr. 10 mo.	32 oz. milk, egg, etc.....	39.3	11.5	41.5	32.3	26.2	
324	T. R.	4 yr.	32 oz. milk, egg, meat, etc...	38.3	13.5	36.9	32.6	30.5	
322	M. J. 1	3 yr.	24 oz. milk, egg, meat, nut butter, etc.....	48.2	12.1	30.0	37.5	32.5	
290	D. R.	3 yr.	22 oz. milk, nut butter, egg, meat, etc.....	44.8	10.5	30.0	17.3	52.7	
296	E. M. 1	2 yr. 3 mo.	32 oz. milk, egg, etc.....	38.8	16.1	28.9	37.5	33.6	
311	R. K. 2	1 yr. 11 mo.	27 oz. milk, nut butter, etc...	31.5	16.9	28.9	47.6	23.5	
309	H. F. 7	1 yr. 2 mo.	20 oz. milk, butter, egg, etc...	39.8	21.0	27.1	29.3	43.6	
Average .....				40.9	20.1	47.9	26.5	25.6	

Table 3 includes the analyses of forty-one normal stools of children whose diet contained considerable amounts of milk. In only three instances (Nos. 345, 348, 350) was the daily quantity less than one

TABLE 3.

*Fat Per Cent. and Distribution in Stools of Normal Children on a Mixed Diet.  
Stools Normal.*

No.	Case	Age	Diet	Intake of Fat, Grams Daily	Analyses of Stools				
					Fat per Cent. of Dried Weight	Per Cent. of Total Fat as			
						Soap	Free Fatty Acids	Neutral Fat	
292	H. M.	1 yr. 6 mo.	30 oz. milk, egg, etc.....	39.3	27.4	78.6	8.3	13.1	
300	F. B. 1	3 yr. 8 mo.	32 oz. milk, egg, meat, etc....	34.3	24.4	70.2	11.9	17.9	
282	B. W. 5	1 yr. 11 mo.	30 oz. skimmed milk, egg, C.L.O., etc.....	23.3	17.3	67.5	15.4	17.1	
281	B. W. 4	1 yr. 10 mo.	40 oz. skimmed milk, egg, C.L.O., etc.....	27.1	20.4	63.7	15.3	21.0	
267	O. W. 1	2 yr. 6 mo.	32 oz. milk, egg, meat, etc....	37.8	18.0	57.0	22.3	20.7	
291	R. K.	2 yr. 1 mo.	27 oz. milk, egg, butter, etc....	42.5	31.2	55.2	19.1	25.7	
203	F. B. 4	3 yr. 7 mo.	20 oz. skimmed milk, butter, egg, meat, etc.....	41.6	26.6	55.1	23.5	21.4	
276	R. L. 2	3 yr.	24 oz. milk, nut butter, egg, meat, etc.....	48.1	10.0	54.5	27.2	18.3	
353	R. L. 5	4 yr. 2 mo.	16 oz. milk, butter, egg, etc....	40.0	20.7	54.1	22.3	23.6	
351	F. B. 5	3 yr. 9 mo.	19.5 oz. skimmed milk, butter, egg, etc.....	44.4	20.4	53.5	17.2	29.3	
254	E. M. 6	2 yr. 7 mo.	19.5 oz. fat-free milk, butter, egg, etc.....	37.7	16.7	52.6	22.6	24.8	
345	E. H.	3 yr. 4 mo.	12 oz. milk, butter, meat, etc....	24.5	8.7	52.1	19.9	28.0	
314	P. F.	3 yr. 11 mo.	20 oz. milk, butter, meat, etc....	36.3	13.9	50.8	20.0	29.2	
260	D. S. 2	5 yr.	32 oz. milk, egg, meat, C.L.O., etc.....	59.0	29.1	50.6	28.7	20.7	
352	F. B. 7	3 yr. 9 mo.	19.5 oz. fat-free milk, butter, egg, meat, etc.....	41.1	20.7	49.9	21.2	28.9	
264	F. W. 9	3 yr. 7 mo.	20 oz. milk, egg, meat, nut butter, etc.....	43.6	16.3	49.8	37.1	13.1	
275	R. L. 1	3 yr.	24 oz. milk, butter, egg, meat, etc.....	48.1	15.4	48.0	32.4	19.6	
259	D. S. 1	5 yr.	32 oz. milk, egg, meat, C.L.O., etc.....	58.5	22.5	49.2	33.5	17.3	
313	E. K.	2 yr.	32 oz. milk, egg, meat, etc....	37.8	17.6	42.2	30.3	27.5	
319	J. O. 1	3 yr.	26 oz. milk, nut butter, etc....	30.5	39.1	41.5	39.3	19.2	
274	A. W. 5	4 yr. 2 mo.	24 oz. milk, nut butter, egg, meat, etc.....	52.1	13.6	41.4	31.6	27.0	
289	M. C.	6 yr.	32 oz. milk, eggs, meat, etc....	44.5	16.6	41.4	34.1	24.5	
312	R. K. 3	2 yr.	32 oz. milk, egg, meat, etc....	38.3	12.4	41.4	28.5	30.1	
287	W. W.	2 yr. 5 mo.	29 oz. milk, butter, egg, meat, etc.....	50.9	14.8	41.2	21.6	37.2	



TABLE 3—*Concluded.*

No.	Case	Age	Diet	Intake of Fat, Grams Daily	Analyses of Stools				
					Fat per Cent. of Dried Weight	Per Cent. of Total Fat as			
						Soap	Free Fatty Acids	Neutral Fat	
330	H. F.	4 yr. 6 mo.	32 oz. milk, egg, meat, etc....	38.3	11.6	41.2	34.2	24.6	
354	R. L. 7	4 yr. 3 mo.	19 oz. skimmed milk, butter, egg, etc.....	43.2	16.7	41.0	26.4	32.6	
271	A. W. 2	4 yr. 1 mo.	24 oz. milk, nut butter, egg, meat, etc.....	48.1	17.6	40.9	32.1	27.0	
293	E. S.	1 yr. 2 mo.	19 oz. milk, butter, egg, etc....	30.9	18.7	40.7	29.8	29.5	
272	A. W. 3	4 yr. 1 mo.	24 oz. milk, egg, corn oil, meat, etc.....	52.1	20.6	40.4	33.2	26.4	
298	E. M. 3	2 yr. 6 mo.	20 oz. milk, nut butter, egg, meat, etc.....	34.1	13.3	37.2	38.3	24.5	
273	A. W. 4	4 yr. 1 mo.	24 oz. milk, egg, corn oil, meat, etc.....	52.1	20.6	36.2	33.8	30.0	
299	E. M. 4	2 yr. 6 mo.	20 oz. skimmed milk, egg, meat, etc.....	40.1	17.4	35.6	33.1	31.3	
297	E. M. 2	2 yr. 4 mo.	20 oz. milk, butter, egg, meat, etc.....	32.6	16.0	35.3	31.7	33.0	
295	M. M.	5 yr. 6 mo.	32 oz. milk, egg, meat, etc....	38.8	11.7	34.4	39.2	26.4	
302	F. B. 3	3 yr. 9 mo.	24 oz. milk, nut butter, egg, meat, etc.....	42.5	21.0	33.6	27.7	38.7	
317	K. P.	2 yr. 8 mo.	21 oz. milk, nut butter, etc....	34.5	9.7	33.0	31.5	35.5	
355	R. L. 9	4 yr. 3 mo.	19 oz. fat-free milk, butter, egg, etc.....	44.6	15.5	32.3	23.5	44.2	
288	E. v. S.	5 yr. 7 mo.	17 oz. milk, butter, egg, meat, etc.....	49.9	9.6	31.6	31.1	37.3	
350	H. J. 2	1 yr. 4 mo.	12 oz. milk, nut butter, meat, etc.....	29.1	14.9	30.0	36.5	33.5	
325	C. M.	5 yr.	16 oz. milk, butter, egg, meat, etc.....	36.8	11.3	24.0	31.8	44.2	
348	M. J. 3	3 yr. 4 mo.	14 oz. milk, nut butter, egg, meat, etc.....	31.3	19.5	19.4	24.6	56.0	
Average .....				40.5	18.0	45.1	27.4	27.5	

pint. These stools were all from children in good condition, and the findings may be taken as being fairly representative of what is normal with a general mixed diet. We have regarded as normal a stool which

was smooth, homogeneous, not hard, showing no mucus or undigested food. The reaction to litmus of the fresh stools in the groups reported in this table and the two previous ones was in the majority of cases decidedly alkaline; in a few it was amphoteric; in no case was it distinctly acid.

The average fat percentage of dried weight and the distribution of fat of the normal stools, as shown in Table 3, are practically the same as in the constipated stools of children on a similar diet. This corresponds to the results obtained with stools of infants whose diet was milk modifications, as published in a previous paper. With such infants no difference was shown between normal and constipated stools in the proportions of fat, protein and ash. The chief difference seems to be in the water content. Since with children on a mixed diet, also, the fat percentage of dried weight averaged nearly the same in the constipated and the normal stools, the values for total ash for each kind of stools were averaged. In the constipated stools the average ash was found to be 19.8 per cent. of the dried weight and in the normal stools it was 18.7 per cent. Although the protein percentage was not determined, the similarity between the constipated and normal stools in both fat and ash percentage confirms our belief that the chief difference between the constipated and normal stools, with mixed diet as with simple milk feeding, is in the water content, and that the chemical composition of the stool has no constant relation to the constipation.

In Table 4 are shown the analyses of a group of stools not normal. All were acid in reaction, some extremely so. All but one was distinctly abnormal in appearance; many showed considerable mucus; almost all contained undigested food and several showed evidences of fermentation. The children nearly all had milk in varying amounts, but in over half the cases the amount of milk consumed was less than one pint daily. However, the average total fat intake was but little less than that seen in Tables 2 and 3.

We have called attention in a previous paper to the fact that in the process of drying the acid stools of nursing infants, a change takes place in the relation between the fatty acids and the soaps, probably owing to the volatilization of lower acids, such as acetic and lactic.<sup>2</sup>

TABLE 4.

*Fat Per Cent. and Distribution in Stools of Normal Children on a Mixed Diet.  
Stools Not Normal.*

No.	Case	Age	Diet	Intake of Fat, Grams Daily	Analyses of Stools				
					Fat per Cent. of Dried Weight	Per Cent. of Total Fat as			
						Soap	Free Fatty Acids	Neutral Fat	
279	B. W. 2	1 yr. 4 mo.	30 oz. milk, egg, C.L.O., etc.	41.6	33.4	58.7	28.2	13.1	
285	B. W. 13	2 yr. 4 mo.	28 oz. milk, nut butter, egg, etc.	51.9	18.6	50.2	26.1	23.7	
301	F. B. 2	3 yr. 9 mo.	24 oz. milk, butter, egg, meat, etc.	43.4	21.6	49.5	20.4	30.1	
328	D. D.	1 yr. 8 mo.	24 oz. milk, meat, C.L.O., etc.	41.3	12.0	48.9	35.2	15.9	
247	B. M.	4 yr.	6.5 oz. fat-free buttermilk, butter, bacon, etc.	35.6	6.7	45.0	26.3	28.7	
286	B. W. 14	2 yr. 4 mo.	28 oz. milk, egg, nut butter, etc.	51.9	20.9	44.6	24.5	30.9	
248	E. D.	2 yr. 6 mo.	11.5 oz. fat-free buttermilk, much cereal, vegetable, etc.	9.7	20.4	41.4	47.8	10.8	
344	K. v. S.	2 yr. 7 mo.	10 oz. milk, butter, egg, meat, etc.	37.7	8.7	33.9	29.1	37.0	
346	E. P.	.....	7 oz. milk, butter, meat, egg, etc.	29.5	14.7	33.7	36.1	30.2	
340	L. S.	3 yr. 2 mo.	10 oz. milk, egg, meat, fish, etc.	30.5	8.2	32.8	27.1	40.1	
339	E. A. 1	7 yr.	15 oz. milk, butter, meat, etc.	32.2	11.3	32.5	26.8	40.7	
349	H. J. 1	1 yr. 4 mo.	15 oz. milk, nut butter, egg, meat, etc.	35.5	12.9	31.8	37.7	30.5	
327	R. M.	4 yr.	32 oz. milk, eggs, meat, C.L.O., etc.	50.5	17.1	28.2	44.7	27.1	
338	R. N.	5 yr. 10 mo.	No milk, butter, meat, much vegetable, etc.	23.9	4.3	28.1	40.8	31.1	
277	R. L. 3	3 yr. 1 mo.	24 oz. milk, nut butter, egg, etc.	52.1	8.4	20.8	40.7	38.5	
329	H. D.	5 yr. 10 mo.	32 oz. milk, egg, meat, etc.	37.8	6.4	16.5	29.6	53.9	
336	R. K.	6 yr.	13 oz. milk, butter, egg, meat, etc.	45.6	14.9	12.9	34.9	52.2	
347	M. J. 2	3 yr.	12 oz. milk, nut butter, egg, meat, etc.	32.4	34.3	9.3	8.9	81.8	
337	D. K.	4 yr.	13 oz. milk, butter, egg, meat, etc.	46.6	12.2	5.0	29.6	65.4	
Average .....				38.4	15.1	32.8	31.3	35.9	

As a result the soaps are higher and the fatty acids lower in the dried stools than in the moist fresh stools, although the amount of total fat is not altered. In the normal alkaline stools of artificially fed children investigation has shown no difference in the total fat or in the distribution of fat between the moist and the dried stool. In the acid stools, however, such as are considered in Table 4, a different distribution of fat is found in the moist stool from that found in the dried stool. We have not yet determined how constantly and to what extent this change in the relation between fatty acids and soaps takes place in the drying of acid stools or what the factors are which affect the process, but undoubtedly a portion of the free fatty acids is converted into soaps. Hence, the figures in Table 4 show higher soaps and lower fatty acids than would have been found if moist stools had been analyzed.

The fat percentage of dried weight shown in Table 4 averages somewhat lower than in the preceding tables, and there is a larger number of cases with low values. The neutral fat averages considerably higher than in normal stools, and there are several high values, suggesting that when the stools are of this character there is a lessened degree of fat splitting. The average for fatty acids is also higher than that found in normal stools. When it is considered that this value would be increased if determined in the moist stool, it is evident that in the average for these acid stools fatty acids form the predominating part of the total fat, the soap being the lowest fraction.

It is to be noted that in eleven of the nineteen cases in Table 4 the intake of calcium and other salts was low because of the small quantity of milk taken, the amount of calcium in the other foods being small in comparison with that in milk. The intake of salts, particularly those of calcium, may have some significance in determining the character of the stools, especially affecting the distribution of the fat.

In Table 5 is given a comparison of the averages of Tables 1, 2 and 3 with the results obtained on normal and constipated stools of infants fed on milk modifications, as reported in a previous paper.<sup>1</sup>

This table shows strikingly the difference in fat percentage and distribution of fat between the stools when the diet contained a considerable proportion of solid food and those when the diet was mainly milk, both the total fat percentage and the soap percentage being much



lower in the stools of children on a mixed diet. Here, also, is shown the close agreement, previously mentioned, in the composition of the normal and the constipated stools when the diet is similar.

In no group studies was there found any constant relation between the amount of the fat intake and either the fat percentage of dried weight or the distribution of fat in the stools.

TABLE 5.

*Comparison between Average Analyses of Stools of Infants and Older Children.*

Food	Character of Stools	Intake of Fat, Grams Daily	Analyses of Stools			
			Fat per Cent. of Dried Weight	Per Cent. of Total Fat as		
				Soap	Free Fatty Acids	Neutral Fat
Infants on usual milk modifications	Normal.....	24.0	36.2	72.8	16.5	9.4
	Constipated.....	22.9	36.0	73.8	17.6	9.0
Children on milk or milk with bread and cereal	Normal or constipated....	29.6	30.7	60.9	25.1	14.0
Children on mixed diet	Normal.....	40.5	18.0	45.1	27.4	27.5
	Constipated.....	40.9	20.1	47.9	26.5	25.6

## 2. Fat Retention of Normal Children.

Table 6 shows the fat excretion and retention of the children taking only milk or milk with bread and cereal. The stools were normal in all but one instance (No. 229), when they were constipated.

The average fat loss shown in Table 6 is 2.76 gm. The percentage of the intake retained averages 90.6, a value corresponding closely to that found for infants receiving milk modifications.

The fat excretion and the fat retention of children taking a mixed diet are shown in Tables 7, 8 and 9. Table 7 shows the values when the stools were constipated, Table 8 when they were normal, and Table 9 when they were acid abnormal stools.

The average excretion and retention are seen to be almost identical in Tables 7 and 8. The average fat loss when the stools were constipated is 2.51 per cent.; when they were normal, 2.48 per cent. The

TABLE 6.

*Fat Retention of Normal Children on a Diet of Milk or Milk with Bread and Cereal.  
Stools Normal or Constipated. (Cf. Table 1.)*

No.	Case	Intake of Fat, Gm. Daily	Fat in Stools, Gm. Daily	Fat Retained, Gm. Daily	Per Cent. of Intake Retained
225	C. A. 1	35.0	4.10	30.9	88.3
229	M. S.	32.0	1.88	30.1	94.2
233	F. W. 8	31.0	4.10	26.9	86.8
232	J. E.	28.0	1.92	26.1	93.2
234	A. L.	21.8	1.81	20.0	91.8
Average.....		29.6	2.76	26.8	90.6

TABLE 7.

*Fat Retention of Normal Children on a Mixed Diet; Stools Constipated. (Cf. Table 2.)*

No.	Case	Intake of Fat, Gm. Daily	Fat in Stools, Gm. Daily	Fat Retained, Gm. Daily	Per Cent. of Intake Retained
321	F. S.	61.0	2.46	58.5	96.1
280	B. W. 3	55.5	4.57	50.9	91.7
269	O. W. 5	53.5	3.20	50.3	94.0
322	M. J. 1	48.2	1.04	47.2	97.8
290	D. R.	44.8	1.26	43.5	97.2
283	B. W. 6	42.1	3.36	38.7	92.0
284	B. W. 12	40.5	3.85	36.6	90.5
309	H. F. 7	39.8	2.71	37.1	93.0
296	E. M. 1	38.8	2.29	36.5	94.1
268	O. W. 2	38.5	2.16	36.3	94.4
270	A. W. 1	38.4	1.43	37.0	96.3
320	J. O. 2	38.3	1.20	37.1	96.8
324	T. R.	38.3	1.49	36.8	96.1
307	H. F. 3	36.6	2.55	34.0	92.9
308	H. F. 5	35.2	1.96	33.2	94.3
305	H. F. 1	34.9	1.68	33.2	95.2
326	E. A. 2	34.0	4.17	29.8	87.7
318	D. L.	33.6	4.04	29.6	88.0
311	R. K. 2	31.5	2.36	29.1	92.5
Average.....		41.2	2.51	38.7	93.9

TABLE 8.

*Fat Retention of Normal Children on a Mixed Diet; Stools Normal. (Cf. Table 3.)*

No.	Case	Intake of Fat, Gm. Daily	Fat in Stools, Gm. Daily	Fat Retained, Gm. Daily	Per Cent. of Intake Retained
260	D. S. 2	59.0	4.71	54.3	92.1
259	D. S. 1	58.5	5.65	52.8	90.4
272	A. W. 3	52.1	2.20	49.9	95.8
273	A. W. 4	52.1	5.07	47.0	90.3
274	A. W. 5	52.1	2.56	49.5	95.2
287	W. W.	50.9	2.40	48.5	95.3
288	E. v. S.	49.9	2.22	47.7	95.7
276	R. L. 2	48.1	1.82	46.3	96.3
271	A. W. 2	48.1	1.21	46.9	97.5
355	R. L. 9	44.6	3.34	41.3	92.6
289	M. C.	44.5	1.70	42.8	96.2
351	F. B. 5	44.4	2.74	41.7	93.8
264	F. W. 9	43.6	2.92	40.7	93.3
354	R. L. 7	43.2	3.29	39.9	92.4
291	R. K.	42.5	2.18	40.3	94.8
302	F. B. 3	42.5	2.25	40.2	94.6
352	F. B. 7	41.1	2.85	38.3	93.2
299	E. M. 4	40.1	2.53	37.6	93.7
353	R. L. 5	40.0	3.57	36.4	91.2
292	H. M.	39.3	2.50	36.8	93.6
295	M. M.	38.8	1.56	37.2	95.9
312	R. K. 3	38.3	0.95	37.3	97.5
330	H. F.	38.3	1.62	36.7	95.8
313	E. K.	37.8	3.57	34.2	90.6
267	O. W. 1	37.8	1.89	35.9	95.0
254	E. M. 6	37.7	2.94	34.8	92.2
325	C. M.	36.8	1.35	35.4	96.3
314	P. F.	35.3	1.26	35.0	96.6
317	K. P.	34.5	1.23	33.3	96.4
300	F. B. 1	34.3	2.16	32.1	93.6
298	E. M. 3	34.1	2.53	31.6	92.6
297	E. M. 2	32.6	1.95	30.6	94.0
348	M. J. 3	31.3	2.06	29.2	93.3
350	H. J. 2	29.1	1.35	27.7	95.4
281	B. W. 4	27.1	3.53	23.6	87.2
245	E. H.	24.5	1.06	23.4	95.7
282	B. W. 5	23.3	2.92	20.4	87.7
Average .....		40.8	2.48	38.3	93.9

percentage of the intake retained is 93.9 for both groups, with only two values in each table below 90. This shows how exceedingly good is the fat retention of most children on a mixed diet. It is also noteworthy that the fat loss in constipated stools is no greater than that in normal stools.

The values for intake, excretion and retention in Table 9 show surprisingly little variation either in range or average from those

TABLE 9.

*Fat Retention of Normal Children on a Mixed Diet; Stools Not Normal. (Cf. Table 4.)*

No.	Case	Intake of Fat, Gm. Daily	Fat in Stools, Gm. Daily	Fat Retained, Gm. Daily	Per Cent. of Intake Retained
277	R. L. 3	52.1	1.18	50.9	97.5
285	B. W. 13	51.9	5.33	46.6	89.8
286	B. W. 14	51.9	3.70	48.2	93.0
327	R. M.	50.5	4.35	46.1	91.4
337	D. K.	46.6	1.31	45.3	97.2
336	R. K.	45.6	1.62	44.0	96.5
301	F. B. 2	43.4	3.53	39.9	92.0
279	B. W. 2	41.6	4.33	37.3	89.7
328	D. D.	41.3	1.63	39.7	96.1
344	K. v. S.	37.7	1.05	36.6	97.3
247	B. M.	35.6	0.71	34.9	98.0
349	H. J. 1	35.5	1.10	34.4	96.9
347	M. J. 2	32.4	4.44	28.0	86.3
339	E. A. 1	32.2	1.51	30.7	95.3
340	L. S.	30.5	1.58	28.9	94.9
346	E. P.	29.5	1.59	27.9	94.7
338	R. N.	23.9	1.43	22.5	94.1
Average .....		40.1	2.38	37.7	94.0
248	E. D.	9.7	4.42	5.3	54.3

found when the stools were normal or constipated. For percentage of intake retained there are only four values below 90.

In observation 248 the intake was so unusually low that the figures are not included in the average. The diet in this case consisted largely of carbohydrates (67.9 per cent. of the total calories). Although the fat intake was so low, the excretion of fat was as high as that of some children whose intake was five times as great. The stools showed much starch and vegetable waste.



It is to be noted that the acid abnormal stools reported in Table 9 were not watery stools. These very acid stools, however, may possibly be one of the early indications of a serious digestive disturbance, which might end in diarrhea with a high loss of fat in the stools. That an excessive fat loss with consequently lowered retention occurs as a result of diarrhea was clearly shown with our breast fed and artificially fed infants. Very loose stools, however, are not frequently seen with older children on a mixed diet, unless the children are acutely ill, and no such cases are included in this study.

Table 10 gives a comparison of the fat excretion and retention of normal infants on milk modifications with the excretion and retention of normal children on a mixed diet.

TABLE 10.

*Comparison of Average Fat Retention of Infants and Older Children.*

Food	Character of Stool	Intake of Fat, Gm. Daily	Fat in Stools, Gm. Daily	Fat Retained, Gm. Daily	Per Cent. of Intake Retained
Infants on usual milk modifications	Constipated . . . . .	27.1	2.74	24.4	89.9
	Normal . . . . .	26.7	2.33	24.4	91.3
Children on milk or milk with bread and cereal	Normal or constipated . . . . .	29.6	2.76	26.8	90.6
Children on mixed diet	Constipated . . . . .	41.2	2.51	38.7	93.9
	Normal . . . . .	40.8	2.48	38.3	93.9
	Not normal . . . . .	40.1	2.38	37.7	94.0

The most striking point brought out in Table 10 is the uniformity in the daily fat excretion. The average values show little difference whatever the kind of diet or the amount of fat intake. It is surprising that older children with a much higher intake lost practically the same amount of fat as did the infants. This results in a higher percentage retention for the older children. Here again is emphasized the fact that the retention of fat is the same when the stools were constipated as when they were of normal consistency.

The second part of the paper deals with two groups of children not in normal condition as to digestion. In the first group are considered the rachitic cases; in the second are considered the children suffering from chronic intestinal indigestion, in several cases with intestinal infantilism.

3. *Fat Content and Distribution in Stools of Rachitic Children.*

In Table 11 are given the analyses of the stools which were normal or constipated, and in Table 12 are given the analyses of those which were acid in reaction and showed undigested food or evidence of fermentation.

TABLE 11.

*Fat Per Cent. and Distribution in Stools of Rachitic Children on a Mixed Diet. Stools Normal or Constipated.*

No.	Case	Age	Diet	Intake of Fat, Grams Daily	Analyses of Stools				
					Fat per Cent. of Dried Weight	Per Cent. of Total Fat as			
						Soap	Free Fatty Acids	Neutral Fat	
262	F. W. 2	3 yr. 2 mo.	35 oz. milk, meat, etc.....	33.8	20.1	72.0	13.3	14.7	
316	L. R. 2	1 yr. 11 mo.	40 oz. milk, egg, etc.....	52.5	32.0	64.3	12.8	22.9	
265	I. H. 1	2 yr. 6 mo.	32 oz. milk, egg, meat, C.L.O., etc.....	58.3	30.4	63.2	21.4	15.4	
315	L. R. 1	1 yr. 10 mo.	34 oz. milk, egg, etc.....	39.8	25.4	62.6	15.5	21.9	
261	F. W. 1	3 yr. 1 mo.	30 oz. milk, meat, etc.....	24.2	23.7	61.4	22.1	16.5	
262	F. W. 3	3 yr. 4 mo.	32 oz. milk, egg, meat, C.L.O., etc.....	56.7	32.1	59.6	19.7	20.7	
256	R. M. 2	2 yr. 7 mo.	29 oz. milk, eggs, C.L.O., etc...	55.7	50.7	48.8	35.8	15.4	
258	R. M. 4	2 yr. 10 mo.	30 oz. milk, egg, olive oil, etc...	47.3	44.9	45.4	25.7	28.8	
257	R. M. 3	2 yr. 8 mo.	27 oz. milk, eggs, butter, etc...	61.0	36.9	44.7	26.8	28.5	
266	I. H. 2	2 yr. 8 mo.	32 oz. milk, egg, meat, C.L.O., etc.....	46.9	20.6	40.6	36.8	22.6	
Average .....				47.6	31.7	56.3	23.0	20.7	

The stools considered in Table 11 were all alkaline to litmus, and in Nos. 261, 256 and 258 were constipated. It is noteworthy that the diet in every case contained a large amount of milk, the smallest amount being 27 ounces daily. The average fat intake is higher than that of any group of the normal children on mixed diet because of the addition of cod liver oil or other fat for therapeutic purposes.

The fat percentage of dried weight is distinctly higher than that found for children in normal condition. The soap percentage of total fat is higher and the neutral fat and fatty acids correspondingly lower

than in similar stools of normal children. The stools which were constipated show no higher values for soap than do the others.

The stools considered in Table 12 were all acid and showed undigested food, mucus or evidences of fermentation. In this group the amount of milk taken was much less than in the preceding group, the largest amount shown in this table being less than the smallest seen in Table 11. There is a possible connection between the small intake of milk and the acid abnormal stools. This was previously suggested

TABLE 12.

*Fat Per Cent. and Distribution in Stools of Rachitic Children on a Mixed Diet. Stools Not Normal.*

No.	Case	Age	Diet	Intake of Fat, Grams Daily	Analyses of Stools			
					Fat per Cent. of Dried Weight	Per Cent. of Total Fat as		
						Soap	Free Fatty Acids	Neutral Fat
304	R. A. 1	4 yr.	24 oz. milk, butter, egg, meat, etc.....	42.1	23.3	55.5	24.4	20.1
357	R. A. 3	4 yr. 4 mo.	24 oz. milk, butter, egg, etc....	58.1	18.6	54.3	22.9	22.8
356	R. A. 2	4 yr. 3 mo.	24 oz. milk, butter, egg, etc....	51.5	14.8	44.5	26.2	29.3
253	R. M. 1	2 yr. 5 mo.	12 oz. protein milk, 13 oz. milk, cereal.....	19.6	38.2	43.4	37.3	19.3
235	L. H. 2	1 yr. 6 mo.	26 oz. protein milk, egg, etc....	45.1	32.2	37.8		
333	L. H. 1	1 yr. 5 mo.	19 oz. milk, egg, etc.....	33.5	20.6	19.4		
Average .....				41.7	24.6	42.5	27.7	22.9

in the discussion of Table 9 of this paper. The fat intake shown in Table 12 averages lower than in Table 11, being about the same as that found for the normal children.

The average fat percentage of dried weight is lower than that found in the normal stools of the rachitic children, but higher than that in the stools of normal children. It has been our observation that in stools containing much mucus or waste the fat percentage of dried weight is always lower than in the corresponding group of normal stools. As compared with the normal stools of rachitic children there

is here shown an increase of fatty acids at the expense of soap, which would probably have been more marked if the moist stools had been analyzed, since these stools were all acid in reaction.

#### 4. *Fat Retention of Rachitic Children.*

Tables 13 and 14 show the excretion and retention of fat of these same rachitic children. Table 13 gives the values when the stools were normal or constipated, and Table 14 those when the stools were acid abnormal ones.

TABLE 13.

*Fat Retention of Rachitic Children. Stools Normal. (Cf. Table 11.)*

No.	Case	Intake of Fat, Gm. Daily	Fat in Stools, Gm. Daily	Fat Retained, Gm. Daily	Per Cent. of Intake Retained
257	R. M. 3	61.0	6.42	54.6	89.6
265	I. H. 1	58.3	1.49	56.8	97.3
263	F. W. 3	56.7	3.44	53.3	94.0
256	R. M. 2	55.7	8.59	47.1	84.6
316	L. R. 2	52.5	3.56	48.9	93.0
258	R. M. 4	47.3	7.34	40.0	84.4
266	I. H. 2	46.9	1.58	45.3	96.7
315	L. R. 1	39.8	1.96	37.8	95.0
262	F. W. 2	33.8	2.49	31.3	92.7
261	F. W. 1	24.2	2.97	21.2	87.8
Average .....		47.6	3.98	43.6	91.6

In Table 13 the average daily fat loss, 3.98 gm., although not excessive, is greater than that of normal children on a corresponding diet. Three very high values for fat excretion (Nos. 256, 257, 258) were obtained with the same child (R. M.); excluding these, the average differs little from normal. The percentage of fat intake retained is somewhat lower than that found for normal children, largely because of the values found in the case of R. M. The actual retention is higher than that found for the normal children because of the higher intake.

The average values shown in Table 14 for fat loss and percentage retention are not essentially different from those seen in Table 13. The actual retention is less because of the smaller intake.



The number of cases reported in these tables is too small to permit of any final conclusions, but the findings indicate that rachitic children have a somewhat larger excretion of fat than is usually seen with normal children on a similar diet.

TABLE 14.

*Fat Retention of Rachitic Children. Stools Not Normal. (Cf. Table 12.)*

No.	Case	Intake of Fat, Gm. Daily	Fat in Stools, Gm. Daily	Fat Retained, Gm. Daily	Per Cent. of Intake Retained
357	R. A. 3	58.1	2.33	55.8	96.1
356	R. A. 2	51.5	2.17	49.3	96.0
235	L. H. 2	45.1	5.71	39.4	87.4
304	R. A. 1	42.1	3.61	38.5	91.4
333	L. H. 1	33.5	2.62	30.9	92.3
253	R. M. 1	19.6	4.24	15.4	78.4
Average .....		41.7	3.45	38.2	91.6

#### 5. *Fat Percentage and Distribution in Stools of Children Suffering from Chronic Intestinal Indigestion.*

Tables 15 and 16 present the fat percentage and distribution of fat in the stools of the children suffering from chronic intestinal indigestion. These children represent various types of this condition, but they all had habitually large stools in proportion to the amount of food taken. The observations are divided into two groups according to the character of the stools. The first group is that in which the stools were alkaline or amphoteric. These were usually fairly homogeneous and smooth. All but two (Nos. 341 and 239) were very foul. The other group is that in which the stools were strongly acid, containing mucus—usually in considerable amounts—and showing marked evidences of fermentation.

There was great variety in the diet of the children in these groups. All but one (No. 334) had an unusually low fat provided by milk, either because of a small amount of milk or because the milk was partly or completely skimmed. Several had buttermilk, indicated in the tables as L. A. (lactic acid) milk. In some cases the fat of the diet was increased by the addition of butter or cod liver oil.

The striking point brought out in Table 15, which gives the findings on the alkaline stools, is the high fat percentage of dried weight. The average, 36.4, is very much higher than that found for any group of

TABLE 15.

*Fat Per Cent. and Distribution in Stools of Children Suffering from Chronic Intestinal Indigestion; Diet Mixed; Stools Alkaline.*

No.	Case	Age	Diet	Intake of Fat, Grams Daily	Analyses of Stools				
					Fat per Cent. of Dried Weight	Per Cent. of Total Fat as			
						Soap	Free Fatty Acids	Neutral Fat	
236	G. R. 1	5 yr.	26 oz. fat-free L. A. milk, 2 tbsp. cereal with 4 oz. milk..	12.7	28.0	71.7	18.2	10.1	
237	G. R. 2	5 yr. 8 mo.	9 oz. fat-free L. A. milk, 19 oz. 2% L. A. milk, cereal with 6 oz. milk, egg, etc.....	35.8	47.0	70.7	15.6	13.7	
341	A. E.	2 yr. 9 mo.	10 oz. milk, butter, etc.....	15.5	30.8	70.5	20.4	9.1	
242	F. G. 4	6 yr. 5 mo.	13 oz. fat-free L. A. milk, eggs, 16 oz. 2% L. A. milk, meat, C.L.O., etc.....	33.0	28.2	56.5	15.5	28.0	
241	F. G. 3	6 yr.	29 oz. 2% L. A. milk, nut butter, egg, meat, C.L.O., etc. . .	58.9	38.7	53.1	22.8	24.1	
335	E. R. 1	8 yr. 5 mo.	8 oz. milk, butter, egg, meat, etc.....	29.2	60.5	42.1	31.9	26.0	
332	N. C. 1	6 yr.	36 oz. skimmed milk, egg, meat, etc.....	23.5	21.1	39.8	47.0	13.2	
239	G. R. 4	8 yr.	29 oz. 2% L. A. milk, egg, meat, cereal with 9 oz. milk, C.L.O., etc.....	55.1	28.5	37.6	28.2	34.2	
323	E. R. 2	9 yr. 4 mo.	20 oz. milk, egg, meat, butter, C.L.O., etc.....	48.4	46.5	32.3	43.8	23.9	
331	N. C. 2	6 yr. 4 mo.	36 oz. skimmed milk, egg, meat, C.L.O., etc.....	37.6	34.5	30.5	55.2	14.3	
Average .....				35.0	36.4	50.5	29.9	19.6	

normal children on a mixed diet, and only one value in this table even approximates the normal average. The average percentage of soap is somewhat higher than that found in the stools of normal children. This high value is due, however, to three instances in which the

amounts of solid food were small. The remaining cases show a range and average similar to that found with normal children. The neutral fat is in both range and average rather lower than that for normal children, while the fatty acids are correspondingly higher.

TABLE 16.

*Fat Per Cent. and Distribution in Stools of Children Suffering from Chronic Intestinal Indigestion; Diet Mixed; Stools Acid.*

No.	Case	Age	Diet	Intake of Fat, Grams Daily	Analyses of Stools				
					Fat per Cent. of Dried Weight	Per Cent. of Total Fat as			
						Soap	Free Fatty Acids	Neutral Fat	
244	H. F. 2	3 yr. 3 mo.	16 oz. protein milk, butter, meat, etc.....	39.8	21.2	55.6	27.2	17.2	
238	G. R. 3	7 yr. 8 mo.	22 oz. 2% L. A. milk, cereal with 9 oz. milk, egg, meat, C.L.O., etc.....	49.0	34.8	54.1	19.8	26.1	
294	M. H.	2 yr. 1 mo.	19 oz. milk, meat, butter, etc....	30.5	34.6	42.9	48.2	8.9	
278	W. R. 1	4 yr. 6 mo.	16 oz. milk, nut butter, egg, etc.....	35.2	21.7	42.4	32.4	25.2	
255	D. R.	3 yr.	17 oz. skimmed milk, egg, butter, etc.....	23.8	42.3	49.6	33.1	17.3	
249	S. M.	2 yr. 10 mo.	12 oz. 2% L. A. milk, butter, egg, 12 oz. milk, etc.....	36.7	45.1	36.6	45.0	18.4	
240	F. G. 2	5 yr. 5 mo.	32 oz. 2% L. A. milk, egg, meat, butter, C.L.O., etc....	74.2	59.7	30.3	55.4	14.3	
334	H. L. 1	8 yr.	36 oz. milk, meat, etc.....	33.3	30.8	28.3	52.7	19.0	
342	F. G. 1	5 yr.	No milk, egg, butter, etc.....	21.0	29.8	26.6	33.8	39.6	
243	H. F. 1	2 yr. 10 mo.	32 oz. protein milk (low fat), meat, large amount of carbohydrates, etc.....	17.1	33.3	24.8	65.2	10.0	
Average .....				36.1	35.3	39.1	41.3	19.6	
343	H. L. 2	8 yr.	6 oz. milk, butter, etc. (much potato).....	34.6	19.5	15.6	70.4	14.0	

The fat percentage of dried weight shown in Table 16, in which are reported the findings on the acid stools, is very high for children on a mixed diet. The average, 35.3, is almost the same as that in Table 15.

The chief difference between these findings and those on the alkaline stools of similar children is in the great increase of fatty acids at the expense of soap. This difference would be even greater if the acid stools had been analyzed moist. The neutral fat averages just the same in both types of stools and suggests a good splitting of the ingested fat, which is rather surprising. The values in No. 343 are not included in the average because the stool was mixed with the urine. The exceptionally low value for fat percentage of dried weight in this case is due to the inclusion of the urine solids in the total dried weight. This illustrates the importance of carefully excluding urine in the collection of stools for analysis. The distribution of fat shown in this case is not unusual for this type of stool, but because of the contamination no reliance can be placed on the figures. The observation is included because the retention values, which are reliable, are reported in a succeeding table.

#### *6. Fat Retention of Children Suffering from Chronic Intestinal Indigestion.*

Tables 17 and 18 give the excretion and retention of fat of the children with chronic intestinal indigestion. Table 17 gives the values found when the stools were alkaline or amphoteric; Table 18 those when the stools were distinctly acid.

There is no essential difference in fat excretion and retention whether the stools were alkaline or acid. In both tables is shown a very great loss of fat, the averages being, respectively, 7.29 and 8.03 gm. daily, which is about three times the normal average for children on mixed diet. In six instances the daily fat loss exceeds 10 gm. In only one instance (No. 341) is the loss less than 3.0 gm. This child, A. E., was very small and at the time was taking very little food.

The average percentage of fat retained is 79.1 for the group with alkaline stools and 77.7 for the group with acid stools. With only one of the children referred to in each group is the fat retained as much as 90 per cent. of the fat intake. In the group where the stools were alkaline, a retention of 90.8 per cent. is seen in the case of G. R. 4 (No. 239). The condition of this child, although previously very



TABLE 17.

*Fat Retention of Children Suffering from Chronic Intestinal Indigestion; Stools Alkaline. (Cf. Table 15.)*

No.	Case	Intake of Fat, Gm. Daily	Fat in Stools, Gm. Daily	Fat Retained, Gm. Daily	Per Cent. of Intake Retained
241	F. F. 3	58.9	9.54	49.4	83.9
239	G. R. 4	55.1	5.11	50.0	90.8
323	E. R. 2	48.4	14.64	33.8	69.7
331	N. C. 2	37.6	5.99	31.6	84.1
237	G. R. 2	35.8	11.20	24.6	68.8
242	F. G. 4	33.0	6.19	26.8	81.3
335	E. R. 1	29.2	11.20	18.0	61.6
332	N. C. 1	23.5	3.98	19.5	83.2
341	A. E.	15.5	1.95	13.6	87.8
236	G. R. 1	12.7	3.09	9.6	75.7
Average.....		35.0	7.29	27.7	79.1

TABLE 18.

*Fat Retention of Children Suffering from Chronic Intestinal Indigestion; Stools Acid. (Cf. Table 16.)*

No.	Case	Intake of Fat, Gm. Daily	Fat in Stools, Gm. Daily	Fat Retained, Gm. Daily	Per Cent. of Intake Retained
240	F. G. 2	74.2	18.67	55.5	74.8
238	G. R. 3	49.0	7.49	41.5	84.8
244	H. F. 2	39.8	3.54	36.3	91.1
249	S. M.	36.7	7.30	29.4	80.2
278	W. R. 1	35.2	4.34	30.9	87.8
243	H. L. 2	34.6	8.95	25.6	74.0
334	H. L. 1	33.3	11.34	22.0	66.1
294	M. H.	30.5	4.79	25.7	84.3
255	D. R.	23.8	4.68	19.1	80.2
242	F. G. 1	21.0	4.68	16.3	77.6
243	H. F. 1	17.1	12.50	4.6	26.5
Average.....		35.9	8.03	27.9	77.7

serious, was greatly improved at the time this observation was made. In the group where the stools were acid there is in the case of H. F. 2 (No. 244) a retention of 91.1 per cent. of the intake. This child had since the first observation (cf. H. F. 1, No. 243) been put on a diet in

which the fat was increased from 19 to 33 per cent. of the total calories, with the result that the total fat intake was more than doubled. It is interesting to note that this child with a chronic and very serious indigestion shows with increased fat intake the highest retention in the group, notwithstanding the fact that the stools were still acid. This suggests that the trouble in this case was much more with carbohydrates than with fat.

The actual fat retention of the children with chronic intestinal indigestion varies greatly in the different cases, but in those in which the intake was very high the retention is also high, being quite equal

TABLE 19.

*Comparison of Average Analyses of Stools of Normal Children and Those Suffering from Rickets and from Chronic Intestinal Indigestion.*

Condition of Child	Character of Stools	Intake of Fat, Grams Daily	Analyses of Stools			
			Fat per Cent. of Dried Weight	Per Cent. of Total Fat as		
				Soap	Free Fatty Acids	Neutral Fat
Normal	Normal—alkaline.....	40.5	18.0	45.1	27.4	27.5
	Not normal—acid.....	38.4	15.1	32.8	31.3	35.9
Rachitic	Normal—alkaline.....	47.6	31.7	56.3	23.0	20.7
	Not normal—acid.....	41.7	24.6	42.5	27.7	22.9
With chronic intestinal indigestion	Normal—alkaline.....	35.0	36.4	50.5	29.9	19.6
	Not normal—acid.....	36.1	35.3	39.1	41.3	19.6

to the normal average. This may have an important clinical bearing. The fact that the fat excretion is abnormally high is not in itself a sufficient reason for cutting down the fat in the diet. The really important thing as far as fat metabolism is concerned is the amount of fat retained.

Table 19 gives a comparison of the average analyses of the stools of normal children and those suffering from rickets and from chronic intestinal indigestion.

It is shown in Table 19 that with all three types of children studied the fat percentage of dried weight is lower in the acid stools than in the normal alkaline stools. The fat percentage of dried weight is much

higher in the stools of the abnormal children than in those of the normal children, and is greater in those of the children suffering from chronic intestinal indigestion than in those of the children suffering from rickets.

In each of the groups shown in Table 19 the soap percentage is lower in the acid than in the alkaline stools. In each group also the percentage of fatty acids is greater in the acid stools than in the alkaline, the difference being most marked with children suffering from chronic intestinal indigestion. With normal children the percentage of neutral fat is much higher in the acid stools than in the alkaline, but with the abnormal children there is little or no difference in per-

TABLE 20.

*Comparison of Average Retention of Fat of Normal Children and of Those Suffering from Rickets and from Chronic Intestinal Indigestion.*

Condition of Child	Character of Stools	Intake of Fat, Gm. Daily	Fat in Stools, Gm. Daily	Fat Retained, Gm. Daily	Per Cent. of Intake Retained
Normal	Normal—alkaline...	40.8	2.48	38.3	93.9
	Not normal—acid..	40.1	2.38	37.7	94.0
Rachitic	Normal—alkaline...	47.6	3.98	43.6	91.6
	Not normal—acid..	41.7	3.45	38.2	91.6
With chronic intestinal indigestion	Normal—alkaline...	35.0	7.29	27.7	79.1
	Not normal—acid..	35.9	8.03	27.9	77.7

centage of neutral fat whether the stools were acid or alkaline. The stools of the normal children show a somewhat higher neutral fat and lower soap than do those of the abnormal children. This may not be significant because of the wide range of values shown for the abnormal children and because there was such a wide variety in the diet.

Table 20 compares the average excretion and retention of fat by the normal children and the abnormal children.

Table 20 brings out more clearly the points emphasized in the discussion of the separate retention tables. With all three types of children there is no significant difference in fat excretion or retention, whether the stools were normal alkaline or abnormal acid ones. The fat loss in the stools of the two types of abnormal children is greater

than that of normal children, being somewhat increased with rachitic children and very greatly increased with those suffering from chronic intestinal indigestion. The percentage of the fat intake retained by the rachitic children is but little lower than is usually found in normal cases because of the high intake and because the loss in the stools, although greater than in normal cases, is not excessive. The percentage retention of fat of the children suffering from chronic intestinal indigestion, however, is much lower than is that of normal children, owing to the abnormally large excretion.

#### SUMMARY.

1. In the normal or constipated stools of older children whose diet consisted of milk alone or milk with bread and cereal the fat percentage of dried weight averaged 30.7, which is lower than the average found for similar stools of infants taking modifications of cow's milk. The soap percentage of total fat averaged 60.9, which was somewhat lower than that found in the stools of the infants.

2. The normal and the constipated stools of children on a mixed diet showed almost identical average values both for fat percentage of dried weight and for distribution of fat. The fat percentage of dried weight averaged, respectively, 18.0 and 20.1, and the soap averaged, respectively, 45.1 and 47.9 per cent. of the total fat. These values were much lower than those found when the diet contained little or no solid food.

3. In the acid abnormal stools of children on a mixed diet the fat averaged 15.1 per cent. of the dried weight. Both the fat percentage of dried weight and the soap percentage of total fat were much lower than in normal stools and the values for fatty acids and for neutral fat were higher.

4. With rachitic children the fat percentage of dried weight averaged 31.7 in the alkaline stools, and 24.6 in the acid stools. The values were higher than those found for corresponding types of stools of normal children. The proportions of soap, fatty acids and neutral fat were not significantly different from those for normal children.

5. The stools of children suffering from chronic intestinal indigestion showed a much higher fat percentage of dried weight than those



of normal children; the average for alkaline stools being 36.4 per cent., and for acid stools 35.3 per cent. The average percentage of neutral fat was lower in both alkaline and acid stools of these children than in the stools of normal children. The fatty acids were higher than normal, much higher when the reaction of the stools was acid.

6. The average fat loss in the stools of normal children varied between 2.6 and 3.0 gm. in all the groups studied, being highest in the stools of children whose diet contained the smallest proportion of solid food and the largest proportion of milk.

7. The normal children on mixed diet retained on the average about 94 per cent. of the fat intake, regardless of the type of stool. The average actual retention was about 38 gm. daily. The children with little or no solid food and a smaller fat intake showed a lower actual, and a somewhat lower percentage retention than those on a general mixed diet.

8. The rachitic children showed a slightly larger fat loss in the stools than did the normal children; their intake, however, was higher. Their actual retention, therefore, equalled or exceeded that of the normal children, and their percentage retention was only a little lower than the normal average.

9. The fat loss in the stools of the children suffering from chronic intestinal indigestion was very great, averaging 7.3 gm. daily in the alkaline stools and 8.0 gm. in the acid stools. Both the actual and percentage retention were much lower than normal. The percentage of the intake retained averaged 79.1 when the stools were alkaline and 77.7 when they were acid. When the intake of fat was very high the actual retention was usually as high as that found for normal children.

## FAT METABOLISM OF INFANTS AND YOUNG CHILDREN.

### IV. THE DIGESTION OF SOME VEGETABLE FATS BY CHILDREN ON A MIXED DIET.

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During the last few years there has been a steadily increasing use of vegetable fats, especially nut butter, in the diet of children. This has been due chiefly to the scarcity and the greatly increased cost of milk and butter. Recently, vegetable fats have been used extensively both in the wards and the out-patient department of this hospital. It seemed fitting, therefore, that a study on fat metabolism should include observations on children taking certain of these vegetable fats. While the number of cases on which laboratory studies have been made is not large, enough observations are presented to allow some conclusions to be drawn.

It is not intended in this paper to suggest that vegetable fats are equivalent to milk fat or can entirely replace it in the dietary of children. It is well known that such is not the case. It has been shown by Osborne and Mendel<sup>1</sup> and by McCollum<sup>2</sup> and others<sup>3</sup> that milk fat, as well as some other animal fats, contains a constituent necessary for normal growth and maintenance, to which the name "fat-soluble A" has been given. This is often referred to as the fat soluble vitamin. It has been found not to be present in vegetable fats to any considerable extent. Although this substance is present in certain other foods, especially eggs, and to some extent in the vegetative parts of plants, for example, spinach, lettuce, cabbage, it is evident that these articles

1. Osborne and Mendel: J. Biol. Chem. 16: 423, 1913; *ibid.* 17: 401, 1914.

2. McCollum and Davis: J. Biol. Chem. 15: 167, 1913. McCollum, Simonds and Pitz: Am. J. Physiol. 41: 361 (Sept.) 1916.

3. Halliburton and Drummond: J. Physiol. 51: 235 (Sept.) 1917.

cannot be given to young children in large amounts. Milk and butter are the foods which most easily provide a supply of the fat soluble vitamin ample for the needs of children, for as yet the minimum quantity of this substance required by the growing child has not been determined.

The total fat requirement of children, however, undoubtedly exceeds the amount of milk fat which is needed to supply a sufficient quantity of this vitamin. Vegetable fats, if equally digestible, may therefore be substituted to a considerable degree for milk fat in supplying the fat needed in the diet of children.

Langworthy and Holmes<sup>4</sup> have tested the digestibility of vegetable fats in adults and found that, with the exception of cocoa fat, all those tested, including cocoanut oil and corn oil, were digested quite as well as milk fat. Rockwood and Swickes<sup>5</sup> found that dogs utilized corn oil perfectly well. No reports with laboratory findings of the digestibility of vegetable fats in children have come to our notice.

The observations here reported were made on children who received a mixed diet containing varying amounts of vegetable fat in the form either of nut butter or corn oil. For nut butter several of the brands of nut margarine on the market were used. These margarines consist mainly of cocoanut oil and are labeled as free from animal fat. Cocoanut oil consists mainly of the glycerids of palmitic and oleic acid, in this respect resembling milk fat; but it contains a smaller proportion of olein than does milk fat. Cocoanut oil also contains considerable amounts of myristin and laurin. The melting point of nut butter is slightly lower than that of milk butter, so that it is barely solid at room temperature. This nut butter is white and almost tasteless when fresh, but becomes rancid more easily than does milk butter.

Corn oil is composed largely of the glycerids of palmitic and oleic acid, with a higher proportion of olein than is present in milk fat. It is a liquid, somewhat deeper in color than olive oil. When fresh, it is almost tasteless and odorless, but acquires a somewhat unpleasant taste and odor on continued exposure to the air.

4. Langworthy and Holmes: U. S. Dept. Agric. Bull. 505, 1917. Holmes: U. S. Dept. Agric. Bull. 687, 1918.

5. Rockwood and Swickes: J. A. M. A. 71: 1649 (Nov. 16) 1918.

In order to investigate the digestibility of corn oil and nut butter, analyses were made of the stools of children taking varying amounts of these fats. The feces were collected, dried and analyzed according to the procedure followed in previous investigations of fat in stools.

The first part of this paper gives the fat percentage and distribution of fat in the stools and the retention of fat of these children, arranged in groups according to the proportion of the total fat in the diet which was vegetable fat. The second part of the paper gives for different children the analyses of the stools and the fat retention with various changes in fat intake, both as to the kind of fat and the proportions of vegetable fat and milk fat, together with the principal clinical symptoms with each change.

### *Part I.*

Tables 1 to 7 give the findings in forty-four observations on fourteen children receiving varying proportions of their fat intake in the form of nut butter or corn oil. These children had a general mixed diet. All received milk, usually over one pint daily. In some cases wholly or partially skimmed milk was given in order to reduce the milk fat in the diet. It was necessary, in order to maintain a sufficient intake of salts, especially those of calcium, to use this method of reducing the intake of milk fat instead of diminishing the quantity of milk in the diet. The rest of the food consisted of cereal, bread, vegetables, fruits and usually egg or meat or both.

Tables 1 and 2 give the findings in the instances in which the vegetable fat formed from 20 to 35 per cent. of the total fat intake, Table 1 when the stools were alkaline and Table 2 when they were acid. In the cases in these two tables the vegetable fat was nut butter.

The fat percentage of dried weight in the stools (Table 1) is uniformly low, only two values being higher than the average found for normal stools of children on a mixed diet without significant quantities of vegetable fat. The soap is distinctly lower and the neutral fat higher than when vegetable fat was not given, while the fatty acids are but little different. The fat excretion is unusually low and the percentage of the fat intake retained is slightly higher than was found when the fat of the diet was mainly milk fat.



TABLE 1.

*Fat in Stools and Retention of Fat of Children Receiving from 20 to 35 Per Cent. of the Total Fat Intake as Nut Butter. Stools Alkaline.*

No.	Case	Age	Analyses of Stools				Retention of Fat			
			Fat Percent- age of Dried Weight	Percentage of Total Fat as			Total Fat in Feces, Gm. Daily	Intake of Fat, Gm. Daily	Fat Re- tained, Gm. Daily	Percent- age of Intake Re- tained
				Soap	Free Fatty Acids	Neutral Fat				
276	R. L. 2	3 yrs.	10.0	54.5	27.2	18.3	1.82	48.1	46.3	96.3
275	R. L. 1	3 yrs.	15.4	48.0	32.4	19.6	....	48.1	....	....
271	A. W. 2	4 yrs. 1 mo.	17.6	40.9	32.1	27.0	1.21	48.1	46.9	97.5
298	E. M. 3	2 yrs. 6 mo.	13.3	37.2	38.3	24.5	2.53	34.1	31.6	92.6
302	F. B. 3	3 yrs. 9 mo.	21.0	33.6	27.7	38.7	2.25	42.5	40.3	94.8
317	K. P.	2 yrs. 8 mo.	9.7	33.0	31.5	35.5	1.23	34.5	33.3	96.4
290	D. R.	3 yrs.	10.5	30.0	17.3	52.7	1.26	44.8	43.5	97.2
350	H. J. 2	1 yr. 4 mo.	14.9	30.0	36.5	33.5	1.35	29.1	27.7	95.4
348	M. J. 3	3 yrs. 4 mo.	19.5	19.4	24.6	56.0	2.06	31.3	29.2	93.3
Average.....			14.7	36.3	29.7	34.0	1.71	39.1	37.4	95.7

TABLE 2.

*Fat in Stools and Retention of Fat of Children Receiving from 20 to 35 Per Cent. of the Total Fat Intake as Nut Butter. Stools Acid.*

No.	Case	Age	Analyses of Stools				Retention of Fat			
			Fat Percent- age of Dried Weight	Percentage of Total Fat as			Total Fat in Feces, Gm. Daily	Intake of Fat, Gm. Daily	Fat Re- tained, Gm. Daily	Percent- age of Intake Re- tained
				Soap	Free Fatty Acids	Neutral Fat				
381	H. F. 8	1 yr. 3 mo.	18.9	51.7	23.7	24.6	2.07	43.7	41.6	95.3
285	B. W. 13	2 yrs. 4 mo.	18.6	50.2	26.1	23.7	5.33	51.9	46.6	89.8
278	W. R. 1	4 yrs. 6 mo.	21.7	42.4	32.4	25.2	4.34	35.2	30.9	87.8
286	B. W. 14	2 yrs. 4 mo.	20.9	44.6	24.5	30.9	3.70	51.9	48.2	93.0
349	H. J. 1	1 yr. 4 mo.	12.9	31.8	37.7	30.5	1.10	35.5	34.4	96.9
277	R. L. 3	3 yrs. 1 mo.	8.4	20.8	40.7	38.5	1.18	52.1	50.9	97.5
347	M. J. 2	3 yrs.	34.3	9.3	8.9	81.8	4.44	32.4	28.0	86.3
Average.....			19.4	35.8	27.7	36.5	3.17	43.2	40.0	92.6

In the group in which the stools were acid (Table 2) the fat percentage of dried weight averages somewhat higher than in the alkaline stools. The distribution of fat, which was determined on the dried stools, is almost the same as that found in the alkaline stools, but since the stools were acid the soap values would probably have been lower and the fatty acids higher if the stools had been analyzed in the moist state. These stools were all distinctly abnormal in appearance, showing mucus, undigested food or marked evidences of fermentation. The average total fat excretion is much higher and the percentage of the fat intake retained is lower than when the stools were alkaline.

TABLE 3.

*Fat in Stools and Retention of Fat of Children Receiving from 35 to 70 Per Cent. of the Total Fat Intake as Nut Butter. Stools Alkaline.*

No.	Case	Age	Analyses of Stools				Retention of Fat			
			Fat Percent- age of Dried Weight	Percentage of Total Fat as			Total Fat in Feces, Gm. Daily	Intake of Fat, Gm. Daily	Fat Re- tained, Gm. Daily	Percent- age of Intake Re- tained
				Soap	Free Fatty Acids	Neutral Fat				
384	F. B. 6	3 yrs. 10 mo.	14.4	51.5	20.6	27.9	2.09	40.9	38.8	94.9
264	F. W. 9	3 yrs. 7 mo.	16.3	49.8	37.1	13.1	2.92	43.6	40.7	93.3
361	R. L. 8	4 yrs. 3 mo.	9.7	45.4	23.7	30.9	1.75	45.4	43.7	96.1
274	A. W. 5	4 yrs. 2 mo.	13.6	41.4	31.6	27.0	2.56	52.1	49.5	95.2
379	H. F. 4	1 yr. 1 mo.	20.8	40.2	31.3	28.5	2.56	37.3	34.7	93.1
382	E. M. 5	2 yrs. 6 mo.	13.3	35.9	29.1	35.0	1.84	39.2	37.4	95.3
360	R. L. 6	4 yrs. 2 mo.	10.8	31.0	25.4	43.6	1.86	43.2	41.3	95.7
Average.....			14.1	42.2	28.4	29.4	2.23	43.1	40.9	94.9

The findings when the vegetable fat formed from 35 to 70 per cent. of the total fat intake are given in Tables 3, 4 and 5. In the instances reported in Table 3 the vegetable fat was nut butter, in those in Tables 4 and 5 it was corn oil. None of the stools of the children receiving this proportion of nut butter were acid. In three instances when the fat was corn oil there were acid stools (Table 5).

The fat percentage of dried weight (Table 3) is much the same in range and average as (Table 1) when the diet contained a smaller proportion of nut butter. The average for distribution of fat in the stools is almost the same as when the fat of the diet was chiefly milk

fat. The average fat excretion is slightly less and the percentage retention is slightly higher with this proportion of nut butter than when the fat in the diet was mainly milk fat.

TABLE 4.

*Fat in Stools and Retention of Fat of Children Receiving from 35 to 70 Per Cent. of the Total Fat Intake as Corn Oil. Stools Alkaline.*

No.	Case	Age	Analyses of Stools				Retention of Fat			
			Fat Percentage of Dried Weight	Percentage of Total Fat as			Total Fat in Feces, Gm. Daily	Intake of Fat, Gm. Daily	Fat Retained, Gm. Daily	Percentage of Intake Retained
				Soap	Free Fatty Acids	Neutral Fat				
366	W. R. 3	4 yrs. 7 mo.	26.5	48.6	24.5	26.9	4.66	77.0	72.3	94.0
364	O. W. 4	2 yrs. 7 mo.	19.7	44.1	23.9	32.0	2.10	47.5	45.4	95.6
272	A. W. 3	4 yrs. 1 mo.	20.6	40.4	33.2	26.4	2.20	52.1	49.9	95.8
273	A. W. 4	4 yrs. 1 mo.	20.6	36.2	33.8	30.0	5.07	52.1	47.0	90.3
367	W. R. 4	4 yrs. 7 mo.	24.0	34.8	22.3	42.9	3.87	77.0	73.1	95.0
369	W. R. 6	4 yrs. 9 mo.	12.2	29.1	38.6	32.3	1.50	63.1	61.6	97.6
Average.....			20.6	38.9	29.4	31.7	3.23	61.4	58.2	94.6

TABLE 5.

*Fat in Stools and Retention of Fat of Children Receiving from 35 to 70 Per Cent. of the Total Fat Intake as Corn Oil. Stools Acid.*

No.	Case	Age	Analyses of Stools				Retention of Fat			
			Fat Percentage of Dried Weight	Percentage of Total Fat as			Total Fat in Feces, Gm. Daily	Intake of Fat, Gm. Daily	Fat Retained, Gm. Daily	Percentage of Intake Retained
				Soap	Free Fatty Acids	Neutral Fat				
363	O. W. 3	2 yrs. 7 mo.	16.0	43.1	40.6	16.3	1.31	47.5	46.2	97.2
365	W. R. 2	4 yrs. 7 mo.	14.9	37.7	35.7	26.6	2.57	64.5	61.9	96.0
359	R. L. 4	3 yrs. 1 mo.	27.4	36.4	49.0	14.6	3.94	63.6	59.7	93.8
Average.....			19.4	39.1	41.7	19.2	2.61	58.5	55.9	95.6

When the vegetable fat was corn oil, forming from 35 to 70 per cent. of the total fat intake, the average fat percentage of dried weight in the alkaline stools (Table 4) is slightly higher than that which was

found when the fat of the diet was mainly milk fat. The distribution of fat is similar to that found with milk fat feeding, though the soap is a little lower and the neutral fat correspondingly higher. The fat excretion averages considerably higher than when the diet contained no vegetable fat. The total fat intake is very high, making the actual retention high and the percentage retention about normal.

In the acid stools (Table 5) the fat percentage of dried weight averages about the same as in the alkaline stools when the diet contained the same proportions of corn oil. A difference is seen in the distribution of fat, the fatty acids being considerably increased at the expense of the neutral fat. There is a smaller average fat loss and a slightly higher percentage retention.

TABLE 6.

*Fat in Stools and Retention of Fat of Children Receiving from 70 to 95 Per Cent. of the Total Fat Intake as Nut Butter. Stools Alkaline.*

No.	Case	Age	Analyses of Stools				Retention of Fat			
			Fat Percent- age of Dried Weight	Percentage of Total Fat as			Total Fat in Feces, Gm. Daily	Intake of Fat, Gm. Daily	Fat Re- tained, Gm. Daily	Percent- age of Intake Re- tained
				Soap	Free Fatty Acids	Neutral Fat				
362	R. L. 10	4 yrs. 3 mo.	8.9	47.0	19.9	33.1	1.68	44.6	42.9	96.2
383	E. M. 7	2 yrs. 8 mo.	14.9	43.9	28.0	28.1	2.37	38.1	35.7	93.8
380	H. F. 6	1 yr. 2 mo.	18.4	33.2	37.0	29.8	2.24	35.3	33.1	93.7
385	F. B. 8	3 yrs. 10 mo.	13.3	28.9	21.4	49.7	1.42	41.8	40.4	96.6
Average.....			13.9	38.2	26.6	35.2	1.93	39.9	38.0	95.2

In Tables 6 and 7 are shown the findings when the vegetable fat was 70 to 95 per cent. of the total fat intake. Table 6 gives the values when the fat was nut butter; Table 7 when it was corn oil. The stools were all alkaline, except in one instance with corn oil feeding. The values in this case are placed in the same table with the values for the alkaline stools but are not included in the average.

Table 6 shows that when the diet contained this high proportion of the fat as nut butter the fat percentage of dried weight and the distribution of fat in the stools are not significantly different from that found when the proportion of nut butter was less. The average total



fat in the stools is less and the percentage retention slightly higher than when the fat intake consisted mainly of milk fat.

When the high proportion of corn oil was given the fat percentage of dried weight of the stools (Table 7) is very high, the average being 31.7 per cent. The soap is lower than in any other group in which the vegetable fat formed a significant part of the fat intake. The fat loss is the greatest seen with the vegetable fat feeding, but since the intake is high, the actual retention is still very high and the percentage of the intake retained is good, although lower than in the other groups.

TABLE 7.

*Fat in Stools and Fat Retention of Children Receiving from 70 to 95 Per Cent. of the Total Fat Intake as Corn Oil. Stools Alkaline.*

No.	Case	Age	Analyses of Stools				Retention of Fat			
			Fat Percentage of Dried Weight	Percentage of Total Fat as			Total Fat in Feces, Gm. Daily	Intake of Fat, Gm. Daily	Fat Retained, Gm. Daily	Percentage of Intake Retained
				Soap	Free Fatty Acids	Neutral Fat				
371	F. W. 5	3 yrs. 6 mo.	36.5	37.6	34.9	27.5	5.30	59.4	54.1	91.1
376	B. W. 9	2 yrs. 2 mo.	24.8	37.0	36.3	26.7	....	44.5	....	....
370	F. W. 4	3 yrs. 6 mo.	47.2	34.6	46.9	18.5	4.01	59.4	55.4	93.2
375	B. W. 8	2 yrs. 1 mo.	40.0	34.5	26.6	38.9	7.01	49.8	42.8	86.0
374	B. W. 7	2 yrs. 1 mo.	31.1	32.0	28.5	39.5	6.44	49.7	43.3	87.1
372	F. W. 6	3 yrs. 7 mo.	24.6	21.6	29.7	48.7	3.03	59.4	56.4	94.9
373	F. W. 7	3 yrs. 7 mo.	17.8	14.6	49.7	35.7	3.01	59.4	56.4	94.9
Average.....			31.7	30.3	36.0	33.7	4.80	56.2	51.4	91.5
Stools Acid										
358	C. H.	6 yrs.	13.2	31.8	32.6	35.6	....	35.2	....	....

Table 8 gives a comparison of the averages for the various groups here presented with the averages found when the diet contained no significant proportion of the fat as vegetable fat, both when the stools were normal and when they were acid.

When the vegetable fat in the diet was nut butter in whatever proportion, the fat in the alkaline stools averages about 14 per cent. of the dried weight. In the group of acid stools with nut butter feeding the fat percentage of dried weight is higher than in the alkaline stools,

which is the opposite of what was found with general mixed diet. When a moderate proportion of corn oil was included in the diet, the fat percentage of dried weight in the stools is about the same as when there was no vegetable fat in the diet, whether the stools were acid or alkaline. When, however, the amount of corn oil was large and formed the major part of the fat intake, the fat percentage of dried weight is very high for mixed feeding, averaging 31.7.

TABLE 8.

*Comparison of Fat in Stools and Fat Retention of Children Receiving Milk Fat with That of Children Receiving Varying Proportions of Corn Oil and of Nut Butter.*

Fat in Diet	Reaction of Stools	Analyses of Stools				Retention of Fat			
		Fat Percentage of Dried Weight	Percentage of Total Fat as			Total Fat in Feces, Gm. Daily	Intake of Fat, Gm. Daily	Fat Retained, Gm. Daily	Percentage of Intake Retained
			Soap	Free Fatty Acids	Neutral Fat				
Fat mostly as milk or butter	Alkaline	18.0	45.1	27.4	27.5	2.48	40.8	38.3	93.9
	Acid	15.1	32.8	31.3	35.9	2.38	40.1	37.7	94.0
20 to 35% of fat as nut butter	Alkaline	14.7	36.3	29.7	34.0	1.71	39.1	37.4	95.7
	Acid	19.4	35.8	27.7	36.5	3.17	43.2	40.0	92.6
35 to 70% of fat as nut butter	Alkaline	14.1	42.2	28.4	29.4	2.23	43.1	40.9	94.9
35 to 70% of fat as corn oil	Alkaline	20.6	38.9	29.4	31.7	3.23	61.4	58.2	94.6
	Acid	19.4	39.1	41.7	19.2	2.61	58.5	55.9	95.6
70 to 95% of fat as nut butter	Alkaline	13.9	38.2	26.6	35.2	1.93	39.9	38.0	95.2
70 to 95% of fat as corn oil	Alkaline	31.7	30.3	36.0	33.7	4.80	56.2	51.4	91.5

The distribution of fat in the stools when the diet contained a considerable proportion of vegetable fat shows no striking variation from the findings when the diet did not contain vegetable fat in significant amounts. The soap percentage is usually somewhat lower and the neutral fat somewhat higher. When most of the fat intake was corn oil the soap percentage shows a lower average than that in any of the other groups. The only unusual value for fatty acids is seen

in the group of acid stools with corn oil feeding. Here the fatty acids are much increased at the expense of the neutral fat.

The fat excretion in the alkaline stools when the vegetable fat was nut butter is somewhat less than when the fat intake was mainly milk fat. When the vegetable fat was corn oil the fat loss is greater, being much increased when a large amount of corn oil was given. However, the total fat intake of the children receiving corn oil was very high. In all the groups with vegetable fat feeding the actual retention of fat is never appreciably less than when the diet did not contain vegetable fat and is much greater when the total fat intake was high. In only two groups is the average percentage retention lower than the normal value found with mixed diet without vegetable fat and in these it is over 90 per cent.

### *Part II.*

In order to study the effect on the composition of the stools, the fat retention and the general condition when vegetable fat is substituted for milk fat in varying proportions in a mixed diet, nine children were observed for long periods. In reporting the results of this study each child is considered separately. A table is given which shows the fat percentage and the distribution of fat in the stools and the retention of fat with varying conditions of fat intake, some clinical observations also being included.

These children all received a general mixed diet, usually including an egg daily or alternately egg and meat. The changes in the kind of fat intake are shown in the tables.

*Case 1.*—O. W. The findings in this case showed that when a large proportion of the intake was corn oil the fat retention was excellent and the fat percentage and distribution of the fat in the stools not significantly different from the average found for mixed diet without vegetable fat.

*Case 2.*—A. W. This child showed excellent digestion of vegetable fat, both nut butter and corn oil, the percentage of the intake retained being usually considerably higher than the average found for general mixed diet. In one instance with corn oil feeding there was an unusually large excretion and consequently lower percentage retention, but even in this instance the actual retention was high. The fat percentage and distribution of fat varied little in different observations, whatever the kind of fat.



*Case 3.*—F. W. This child received a diet including 50 gm. of corn oil for a long period. During this time the only sources of fat soluble vitamin were the daily egg and the traces of fat left in the milk, unless, as has been supposed by some, corn oil contains more of this constituent than do the other vegetable oils. The gain in weight and strength continued and the utilization of this large amount of corn oil was good. In observations 370 and 371 the total fat and the fat percentage of dried weight in the stools were unusually large for mixed diet, but with a high intake, the actual retention was much greater than the average for mixed diet without vegetable fat.

*Case 4.*—B. W. In this case also the milk fat in the diet was replaced by a large amount of corn oil. Then egg was omitted from the diet, so that for a considerable period there was no definite source of fat soluble vitamin. The child continued in excellent health and condition, although a week after the egg was omitted from the diet he ceased to gain in weight. During the early part of the long corn oil period he had styes and an attack of urticaria but he was free from these during the later weeks. The stools of this child were habitually larger, whatever the diet, than is usual for his age. The excretion of fat was always high and the percentage of the intake retained never as high as the normal average for mixed diet. Except in the first observations with corn oil feeding, the percentage of the fat intake retained was as good with either corn oil or nut butter as with milk fat. Since with corn oil feeding the soap percentage of total fat in the stools was lower than with milk fat feeding an attempt was made to increase the proportion of soap in the stool by the administration of one ounce of chalk mixture daily. This had very little effect. This child, whose retention of all fats was below normal, showed nearly as good utilization of corn oil and nut butter as of milk fat.

*Case 5.*—W. R. This child before coming under our observation was receiving a diet containing little fat and a large proportion of carbohydrates. He had a chronic intestinal indigestion with acid fermentative stools. When the first observation in this series was made he was taking the diet mentioned. The percentage of the fat intake retained was somewhat below the normal average for mixed diet. The proportion of carbohydrates in the diet was reduced and the fat greatly increased, partly by corn oil. Later both the fat and the protein were increased by adding curd from whole or partially skimmed milk. Although the stools continued to be thin and frequently showed evidences of fermentation, the condition of the child improved and there was a very good gain in weight. The percentage of the fat intake retained, except in one period when he had a severe cold, was higher than the normal average. Although the fat loss in the stools was usually greater than the average for mixed diet, the actual retention was extremely high, because of the unusually large intake of fat. The fat percentage and distribution of fat in the stools showed considerable variation, apparently not related to the kind of fat in the diet. In observation 367 the soap percentage of total fat was low and the neutral fat high. An attempt was made to increase the proportion of soap by administering two ounces of chalk mixture daily and



TABLE 9.  
*Results of Feeding with Corn Oil.*

Case 1.—O. W., aged 2½ years. Healthy child, fairly well nourished. Initial weight, 10,120 gm. Final weight, 10,618 gm.

Period	Fat in Diet	Length of Period, Days	Average Daily Gain or Loss in Weight, Gm.	Appetite	Condition	Stools		No.	Analyses of Stools				Retention of Fat			
						Character	Number Daily		Fat Percentage of Dried Weight	Soap	Free Fatty Acids	Neutral Fat	Total Fat in Feces, Gm. Daily	Intake of Fat, Gm Daily	Fat Retained, Gm. Daily	Percentage of Intake Retained
1	83% of fat as milk	30	+ 4	Good	Improving from slight disturbance of digestion	Good, occasionally hard	1-2	268	29.8	64.6	16.5	18.9	2.16	38.5	36.3	94.4
2	{ 53% of fat as corn oil, 34% of fat as milk (skimmed)	18	+ 27	Good	Excellent	Good, occasionally hard	1-2	364	19.7	44.1	23.9	32.0	2.10	47.5	45.4	95.6
...	.....	17	....	....	Measles, severe attack											
3	{ 28% of fat as cod liver oil, 60% of fat as milk	7	+ 5	Good	Good	Good, occasionally hard	1	269	21.8	53.0	28.2	18.8	3.20	53.5	50.3	94.0

TABLE 10.

Results of Feeding with Corn Oil and Nut Butter.

Case 2.—A. W., aged 4 years. Healthy, well nourished child. Initial weight, 14,830 gm. Final weight, 15,540 gm.

Period	Fat in Diet	Length of Period, Days	Average Daily Gain or Loss in Weight, Gm.	Appetite	Condition	Stools		Analyses of Stools				Retention of Fat			
						Character	Number Daily								
1	83% of fat as milk	19	-36	Good	Good, slightly constipated	Good	0-2	Fat Percentage of Dried Weight	Soap	Free Fatty Acids	Neutral Fat	Total Fat in Feces, Gm. Daily	Intake of Fat, Gm. Daily	Fat Retained, Gm. Daily	Percentage of Intake Retained
2	{ 33% of fat as nut butter, 50% of fat as milk	13	+65	Good	Excellent, slightly constipated	Good	0-1	13.4	47.1	29.7	23.2	1.43	38.4	37.0	96.3
3	{ 38% of fat as corn oil, 46% of fat as milk	24	+24	Good	Excellent	Good, occasionally hard	1	17.6	40.9	32.1	27.0	1.21	48.1	46.9	97.5
...	{ ..... 38% of fat as nut butter, 46% of fat as milk	13	.....	.....	Measles, mild attack			20.6	40.4	33.2	26.4	2.19	52.1	49.9	95.8
4	{ ..... 38% of fat as nut butter, 46% of fat as milk	7	+38	Good	Good, slightly constipated	Constipated	1	20.6	36.2	33.8	30.0	5.07	52.1	47.0	90.3
								13.6	41.4	31.6	27.0	2.56	52.1	49.5	95.2

TABLE 11.

*Results of Feeding with Corn Oil for Long Period with Egg.*

Case 3.—F. W., aged 3 years, 3 months. Well nourished child, previously very rachitic. Initial weight, 9,500 gm. Final weight, 11,120 gm.

Period	Fat in Diet	Length of Period, Days	Average Daily Gain or Loss in Weight, Gm.	Appetite	Condition	Stools		No.	Analyses of Stools				Retention of Fat			
						Character	Number Daily		Fat Percentage of Dried Weight	Percentage of Total Fat as			Total Fat in Feces, Gm. Daily	Intake of Fat, Gm. Daily	Fat Retained, Gm. Daily	Percentage of Intake Retained
1	35% of fat as cod liver oil. 53% of fat as milk Corn oil gradually substituted for milk fat and cod liver oil	31	+23	Fair	Excellent, gaining rapidly in strength  Excellent	Good	1	263	32.1	59.6	19.7	20.7	3.44	56.7	53.3	94.0
2	84% of fat as corn oil (fat-free milk)	46	20+	Fair	Excellent, much more active	Good, occasionally not formed	1-2	370	47.2	34.6	46.9	18.5	4.01	59.4	55.4	93.2
								371	36.5	37.6	34.9	27.5	5.30	59.4	54.1	91.1
								372	24.6	21.6	29.7	48.7	3.03	59.4	56.4	94.9
								373	17.8	14.6	49.7	35.7	3.01	59.4	56.4	94.9
3	37% of fat as nut butter, 46% of fat as milk	9	+44	Good	Measles, mild attack Excellent	Good, sometimes loose	1-2	264	16.3	49.8	37.1	13.1	2.92	43.6	40.7	93.3

TABLE 12.

Results from Replacing Milk Fat by Corn Oil without Egg.

Case 4.—B. W., aged 2 years. Healthy, well nourished child. Initial weight, 10,610 gm. Final weight, 11,710 gm.

Period	Fat in Diet	Length of Period, Days	Average Daily Gain or Loss in Weight, Gm.	Appetite	Condition	Stools		No.	Analyses of Stools				Retention of Fat			
						Character	Number Daily		Fat Percentage of Dried Weight	Percentage of Total Fat as			Total Fat in Feces, Gm. Daily	Intake of Fat, Gm. Daily	Fat Retained, Gm. Daily	Percentage of Intake Retained
1	81% of fat as milk Corn oil gradually substituted for milk fat	25 7	+11 ....	Good ....	Excellent Excellent	Good	1-3	283	24.0	55.5	19.5	25.0	3.36	42.1	38.7	92.0
2	95% of fat as corn oil (fat-free milk)	39	+13	Good	Good, urticaria on legs during two weeks. Inflammation of eyelids appeared twice, developing once into stye	Good	1-2	374 375 376	31.1 40.0 24.8	32.0 34.5 37.0	28.5 26.6 36.3	39.5 38.9 26.7	6.44 7.01 ....	49.7 49.8 44.5	43.3 42.8 ....	87.1 86.0 ....
3	95% of fat as corn oil (fat-free milk) (1 oz. chalk mixture)	13	+11	Good	Excellent	Mushy, then good	1	377 378	20.6 17.5	33.9 40.9	25.2 28.6	40.9 30.5	4.65 3.30	44.5 44.5	39.8 41.2	89.6 92.7
...	.....	15	....	....	Measles, milk attack											
4	79% of fat as milk	8	+29	Good	Excellent	Constipated	1	284	17.8	46.3	26.9	26.8	3.85	40.5	36.7	90.5
5	31% of fat as nut butter, 54% of fat as milk	9	+21	Good	Excellent	Constipated, then acid, not good	1	285	18.6	50.2*	26.1*	23.7	5.33	51.9	46.6	89.8
								286	20.9	44.6*	24.5*	30.9	3.70	51.9	48.2	93.0

\* Stools acid.



TABLE 13.

*Results of Feeding with Corn Oil When Curd Is Included in Diet.*

Case 5.—W. R., aged 4½ years. Fairly well nourished child, suffering from chronic intestinal indigestion. Initial weight, 14,750 gm. Final weight, 15,950 gm.

Period	Fat in Diet	Length of Period, Days	Average Daily Gain or Loss in Weight, Gm.	Appetite	Condition	Stools		No.	Analyses of Stools				Retention of Fat			
						Character	Number Daily		Fat Dried Percentage of	Soap	Free Fatty Acids	Neutral Fat	Total Fat in Feces, Gm. Daily	Intake of Fat, Gm. Daily	Fat Retained, Gm. Daily	Percentage of Intake Retained
1	{ 24% of fat as nut butter, 49% of fat as milk Corn oil gradually added to diet	5	-46	Good	Fair	Fair, acid	0-2	278	21.7	42.4*	32.4*	25.2	4.34	35.2	30.9	87.8
2	{ 54% of fat as corn oil, 31% of fat as milk Corn oil gradually added to diet	22	+11	Good	Good	Loose, fermentative	1-2	365	14.9	37.7*	35.7*	26.6	2.57	64.5	61.9	96.0
3	{ 45% of fat as corn oil, 43% of fat as milk	10	+53	Good	Good, improving	Generally thin with mucus	1-2	366	26.5	48.6	24.5	26.9	4.66	77.0	72.3	94.0
4	{ 42% of fat as corn oil, 44% of fat as milk (2 oz. chalk mixture)	8	-17	Good	Good, except for slight bronchial cold	Thin	1	368	24.0	34.8	22.3	42.9	3.87	77.0	73.1	95.0
5	{ 40% of fat as corn oil, 46% of fat as milk	21	+17	Fair	Improving	Mostly thin	1	369	25.4	54.4	19.3	26.3	4.74	60.1	55.4	92.1
									12.2	29.1	38.6	32.3	1.50	63.1	61.6	97.6

\*Stools acid.

TABLE 14.

*Results of Feeding with Corn Oil and Nut Butter.*

Case 6-A.—R. L., aged 3 years. Neuropathic child, normal as to digestion. Initial weight, 12,050 gm. Final weight, 13,520 gm.

Period	Fat in Diet	Length of Period, Days	Average Daily Gain or Loss in Weight, Gm.	Appetite	Condition	Stools		No.	Analyses of Stools				Retention of Fat			
						Character	Number Daily		Fat Percentage of Dried Weight	Soap	Free Fatty Acids	Neutral Fat	Percentage of Total Fat as	Total Fat in Feces, Gm. Daily	Intake of Fat, Gm. Daily	Fat Retained, Gm. Daily
1	{ 31% of fat as nut butter, 51% of fat as milk 47% of fat as corn oil, 38% of fat as milk	28	+30	Good	Nervous condition improving	Good	0-3	276	10.0	54.5	27.2	18.3	1.82	48.1	46.3	96.3
2		12	+13	Good	Much improved	Good, sometimes thin	1-3	359	27.4	36.4*	49.0*	14.6	3.94	63.6	59.7	93.8

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Case 6-B.—R. L., aged 4 years. Neuropathic child, normal as to digestion. Initial weight, 14,820 gm. Final weight, 14,830 gm.																
1	{ 40% of fat as butter	27	+4	Poor	Fair	Good, sometimes loose	1	353	20.7	54.1	22.3	23.6	3.57	40.0	36.4	91.1
	{ 40% of fat as milk															
2	{ 37% of fat as nut butter,	12	-2	Fair	Improving	Good	1-2	360	10.8	31.0	25.4	43.6	1.86	43.2	41.3	95.7
	{ 44% of fat as milk															
3	{ 60% of fat as butter,	9	+14	Poor	Improving	Good, sometimes soft	1-2	354	16.7	41.0	26.4	32.6	3.29	43.2	39.9	92.4
	{ 21% of fat as milk (skimmed)															
4	{ 57% of fat as nut butter,	7	-8	Fair	Improving	Good, partly not formed	1-2	361	9.7	45.4	23.7	30.9	1.75	45.4	43.7	96.1
	{ 25% of fat as milk (skimmed)															
5	{ 81% of fat as butter (fat-free milk)	9	+67	Good	Good	Good	1-2	355	15.5	32.3	23.5	44.2	3.34	44.6	41.3	92.5
	{ 81% of fat as nut butter (fat-free milk)															
6		7	+8	Good	Good	Good	1	362	8.9	47.0	19.9	33.1	1.68	44.6	42.9	96.2

\* Stools acid.

TABLE 15.

*No Relation Shown to Kind or Amount of Fat.*

Case 7.—E. M., aged 2½ years. Well nourished child, healthy except for general impetigo contagiosa. Initial weight, 12,788 gm. Final weight, 14,505 gm.

Period	Fat in Diet	Length of Period, Days	Average Daily Loss in Weight, Gm. or Gain	Appetite	Condition	Stools		Analyses of Stools				Retention of Fat			
						Character	Number Daily	Fat Percentage of Dried Weight	Soap	Free Fatty Acids	Neutral Fat	Total Fat in Feces, Gm. Daily	Intake of Fat, Gm. Daily	Fat Retained, Gm. Daily	Percentage of Intake Retained
1	84% of fat as milk	13	+37	Fair	Improving	Good	1-2	16.1	28.9	37.5	33.6	2.29	38.8	36.5	94.1
2	24% of fat as butter, 64% of fat as milk	16	+13	Good	Impetigo cured. Abscess on thigh	Good, partly not formed	2-3 then 1	16.0	35.3	31.7	33.0	1.95	32.6	30.6	94.0
3	23% of fat as nut butter, 66% of fat as milk	28	+27	Good	Good, except for short attack of influenza	Good	2-4	13.3	37.2	38.3	24.5	2.53	34.1	31.6	92.6
4	50% of fat as butter, 28% of fat as milk (skimmed)	48	0	Poor	Tonsillitis and influenza during part of period	Good	0-3	17.4	35.6	33.1	31.3	2.53	40.1	37.6	93.7
5	51% of fat as nut butter	9	+37	Fair	Good	Good	1-2	13.3	35.9	29.1	35.0	1.84	39.2	37.4	95.3
...	.....	17	....	....	Tonsils and adenoids removed										
6	80% of fat as butter (fat-free milk)	7	+35	Fair	Fair. Abscess on lip at beginning of period	Good	1	16.7	52.6	22.6	24.8	2.94	37.7	34.8	92.2
7	79% of fat as nut butter (fat-free milk)	6	+17	Good	Good	Mostly good	2-3	14.9	43.9	28.0	28.1	2.37	38.1	35.7	93.8

TABLE 16.

*Results of Feeding with Nut Butter and Milk Butter.*

Case 8.—F. B., aged 3½ years. Well nourished, healthy child. Initial weight, 15,280 gm. Final weight, 15,265 gm.

Period	Fat in Diet	Length of Period, Days	Average Daily Gain or Loss in Weight, Gm.	Appetite	Condition	Stools		No.	Analyses of Stools					Retention of Fat			
						Character	Number Daily		Fat Percentage of Dried Weight	Percentage of Total Fat as				Total Fat in Feces, Gm. Daily	Intake of Fat, Gm. Daily	Fat Retained, Gm. Daily	Percentage of Intake Retained
										Soap	Free Fatty Acids	Neutral Fat					
1	81% of fat as milk	9	+11	Good	Excellent	Good	1-3	300	24.4	70.2	11.9	17.9	2.16	34.3	32.1	93.6	
2	{ 28% of fat as butter, 55% of fat as milk	16	+27	Good	Excellent	Good	1-2	301	21.6	49.5*	20.4*	30.1	3.53	43.4	39.9	91.9	
3	{ 28% of fat as nut butter, 55% of fat as milk	17	+ 3	Poor	Fair, slight attack of influenza, furuncle on leg	Good	1-3	302	21.0	33.6	27.7	38.7	2.25	42.5	40.3	94.8	
4	{ 54% of fat as butter, 26% of fat as milk (skimmed)	7	+86	Good	Influenza and tonsillitis, tonsils and adenoids removed	Good, partly not formed	2	351	20.4	53.5	17.2	29.3	2.74	44.4	41.7	93.8	
5	{ 59% of fat as nut butter, 24% of fat as milk (skimmed)	10	+17	Fair	Otitis media developing	Good, partly not formed	1-2	384	14.4	51.5	20.6	27.9	2.09	40.9	38.8	94.9	
6	{ 83% of fat as butter (fat-free milk)	8	+18	Fair	Fair, otitis media not acute	Good, partly not formed	1-2	352	20.7	49.9	21.2	28.9	2.85	41.1	38.3	93.1	
7	{ 81% of fat as nut butter (fat-free milk)	8	+31	Good	Fair, otitis media becoming acute, inflammation of eyelids, slight skin eruption on upper lip and nostrils	Good	1	385	13.3	28.9	21.4	49.7	1.42	41.8	40.4	96.6	

\* Stools acid.



TABLE 17.

*Results Not Affected by Kind or Amount of Fat.*

Case 9.—H. F., aged 1 year. Healthy child, somewhat under weight. Initial weight, 7,260 gm. Final weight, 9,380 gm.

Period	Fat in Diet	Length of Period, Days	Average Daily Gain or Loss in Weight, Gm.	Appetite	Condition	Stools		No.	Analyses of Stools				Retention of Fat			
						Character	Number Daily		Fat Dried Weight	Percentage of Total Fat as	Free Fatty Acids	Neutral Fat	Total Fat in Feces, Gm. Daily	Intake of Fat, Gm. Daily	Fat Retained, Gm. Daily	Percentage of Intake Retained
1	86% of fat as milk Butter gradually added to diet	19	-13	Good	Fair	Constipated	1-2	305	21.5	53.2	22.7	24.1	1.68	34.9	33.2	95.2
2	49% of fat as butter, 36% of fat as milk (skimmed)	13	....	....	Good	Good	1-3	307	18.9	51.2	29.0	19.8	2.55	36.6	34.1	93.0
3	48% of fat as nut butter, 38% of fat as milk (skimmed)	12	+34	Good	Excellent, slight skin eruption on chin	Good, sometimes hard	1-2	379	20.8	40.2	31.3	28.5	2.56	37.3	34.7	93.1
4	85% of fat as butter (fat-free milk)	16	+8	Good	Fair, slight attack of influenza	Good, sometimes hard	1-2	308	16.3	61.3	18.8	19.9	1.96	35.3	33.3	94.4
5	85% of fat as nut butter (fat-free milk)	14	+27	Fair	Excellent, eruption on chin	Good	0-2	380	18.4	33.2	37.0	29.8	2.24	35.3	33.1	93.7
6	30% of fat as butter, 51% of fat as milk	20	+22	Fair	Good, slightly constipated	Good, partly hard	0-2	309	21.0	27.1	29.3	42.6	2.71	39.8	37.1	93.2
7	28% of fat as nut butter, 55% of fat as milk	17	+9	Fair	Excellent	Constipated, then good	1-2	381	18.9	51.7*	23.7*	24.6	2.07	43.7	41.6	95.3

\* Stools acid.

the next observation showed the highest soap value in the series. Although this child had stools which were looser than normal and frequently fermentative he retained a large proportion of a very high intake, nearly half of which was corn oil.

*Case 6-A.*—R. L. This child showed excellent retention of fat both when nut butter and when corn oil formed part of the fat intake. The percentage of the intake retained was better with nut butter, but with a larger intake the actual retention was better with corn oil. The fat percentage of dried weight in the stools and the total fat excretion were higher with corn oil feeding than when nut butter was taken.

In the succeeding cases the same general plan was followed. A preliminary observation was made with a diet containing a quart of milk but no butter. The amount of milk was then reduced to about 20 ounces and a moderate amount, from 8 to 16 gm., of milk butter was added to the diet. The milk butter was then replaced by an equal amount of nut butter. Partially skimmed milk, with about 1.8 per cent of fat, was then given and a larger amount, from 18 to 26 gm., of butter given, first milk butter and then nut butter. Finally fat-free milk was given with a still larger amount, from 30 to 36 gm., first of milk butter and then of nut butter.

*Case 6-B.*—R. L. In this case two points are to be noted in regard to fat percentage of dried weight in the stools. First, as the proportion of fat in the form of butter increased, the fat percentage of dried weight in the stools decreased. Second, when nut butter replaced the milk butter, the fat percentage of dried weight was much lower than with the same proportion of milk butter. The fat excretion was about twice as great when milk butter was given as when nut butter was given and the percentage of the fat intake retained considerably higher, with the nut butter than with the corresponding amount of milk butter. This child showed excellent digestion of nut butter but there was little or no gain in weight during the periods when nut butter was taken.

*Case 7.*—E. M. With this child the fat excretion was never large and showed no constant relation to the kind or amount of fat. The percentage of the fat intake retained was always fairly high.

*Case 8.*—F. B. In this case, the fat excretion when nut butter was included in the diet was less than the normal average for mixed diet and the percentage of the fat intake retained higher than normal. With milk butter the fat loss was somewhat greater and the percentage of the intake retained slightly lower.

*Case 9.*—H. F. With this child the fat excretion was never large and the percentage of the fat intake retained always good, regardless of the amount and kind of fat in the diet. This child digested milk butter and nut butter equally well.

In general it can be said that in no single instance was there any evidence that the vegetable fats given caused any disturbance of

digestion. No child showed any impairment of appetite. The children took these fats well, in some cases eagerly, and in no case was there vomiting.

The stools of the children taking the large amounts of vegetable fats were, as a rule, rather softer than when mainly milk fat was taken, this being more marked with the corn oil feeding than with nut butter. The frequency of the stools was not increased. In no instance were loose stools seen which could be definitely attributed to the vegetable fat. In the case of W. R., who was suffering from chronic intestinal indigestion, the stools were more loose after the first period, but the fat intake was so greatly increased that the change in the stools cannot be necessarily attributed to the corn oil.

It is to be noted that one child, F. W., took 50 gm. of corn oil daily for forty-six days; another, B. W., took an average of 45 gm. daily for fifty-two days; and a third, W. R., took 35 gm. daily for thirty-two days, all without any disturbing effects.

The increase in weight was, on the whole, quite as uniform and as good with nut butter and corn oil as with milk fat, the only exception being in the case of R. L. (Case 6-B). With this child the decided gain noted in the fifth period was perhaps due to an increase in the amount of food given, coincident with an improvement in the appetite. The marked gain noted following an attack of measles in three instances is to be explained by the fact that during the acute illness the food had been much reduced and the subsequent increase in weight was the gain of convalescence.

#### SUMMARY.

1. The stools of children receiving a considerable proportion of vegetable fat did not differ essentially in appearance from those of children receiving mainly milk fat, although they were usually somewhat softer.

2. The fat percentage of dried weight of the stools averaged somewhat lower when nut butter was taken, and somewhat higher when corn oil was taken, than when the fat in the diet was mainly milk fat; and when large quantities of corn oil were included in the diet the average was much higher.

3. The soap percentage of total fat in the stools was usually a little lower and the neutral fat a little higher with vegetable fat than when the fat of the diet was mainly milk fat.

4. When nut butter was taken the fat excretion in the alkaline stools was lower and in the acid stools it was higher, than when the diet did not contain vegetable fat. When corn oil was taken in considerable amounts the fat excretion in the stools was higher than when the fat of the diet was mainly milk fat. However, the total fat intake when corn oil was included in the diet was very large and the actual retention of fat always much higher than the normal average for mixed diet. When vegetable fat formed a considerable part of the total fat intake, the percentage of the fat intake retained was usually higher than the normal average. In a few instances when the stools were acid and in a few when large amounts of corn oil were taken, the percentage retained was low.

5. The individual children observed for considerable periods with changes in the kind and amount of fat intake showed quite as good digestion of vegetable fat as of corresponding amounts of milk fat and no unfavorable effect on general health and nutrition was observed. No children were kept long enough on a diet presumably deficient in fat soluble A to warrant any conclusions as to the effect of such a diet upon growth and health. In the case of one child who for five weeks was on a diet in which there was no definite source of fat soluble vitamin, 95 per cent. of the fat of the diet being corn oil, he ceased to gain in weight, but showed no loss and the general health continued excellent. The fact may not be without significance that of six children, 80 to 95 per cent. of whose fat intake was vegetable fat, two developed styes and two others eczema upon the face, which disappeared when the diet was changed to include milk fat.

#### CONCLUSION.

The observations published in this paper indicate that corn oil and nut butter, the vegetable fats studied, are valuable foods for children, are exceedingly well borne and are apparently digested and absorbed with ease. We feel, therefore, warranted in the belief that these articles may safely be introduced into the regular diet of children,



and that to a considerable degree they may be substituted for the more expensive milk fat, given either as milk or as butter, but they should never entirely replace milk fat. How much milk fat is needed to furnish the amount of the fat soluble vitamin required for normal growth and nutrition we have not yet the data to determine.

## ETIOLOGY OF THE PNEUMONIAS.\*

By RUFUS COLE, M.D.

*(From the Hospital of The Rockefeller Institute for Medical Research.)*

While certain objections may be raised to employing the term "the pneumonias," I was glad when I was asked to speak in this symposium that this subject was suggested, because it emphasizes a most important point; namely, that pneumonia, even lobar pneumonia, should not be considered a single typical infectious disease.

As you all know, the term "pneumonia" arose first as a clinical term, though with an anatomical significance, which at first, it is true, was mainly assumed. Later, with the development of pathological anatomy the anatomical significance of the term became emphasized so that in its present usage it means first an inflammation of the lungs and only secondarily the reaction of the body to the parasites inciting the inflammatory reaction. In the minds of many, pneumonia still signifies a pathological lesion, not a disease. In comparatively recent years, however, it has been shown that a peculiar group of symptoms and the massive form of diffuse fibrinous inflammation which we call lobar are always associated. We now also know that when this clinical-pathological complex arises there are always present in the lung bacteria of one great group called pneumococcus.

It has been shown, moreover, that not all the pneumococci present in this disease are identical, and by special methods it has been found possible to group them into at least four types or groups. This became important for us as physicians, only as this fact affects our attempts at prevention and cure. It is not very important for us, for instance, that certain strains of diphtheria bacilli show peculiar morphological or cultural characteristics since the reaction of the human body is the same to all strains and since all strains are identical in their immunological reactions; all react to the same serum.

\* Read at the Annual Meeting of the Medical Society of the State of New York, at Syracuse, May 6, 1919.

The conditions are different with pneumococci, however, for each of three of the types is very specific in its immune reactions. Therefore, from the standpoint of specific cure and prevention, we would be justified, if it were possible, in classifying or grouping the cases, using the type of pneumococcus concerned as a basis. The relative frequency of occurrence of the different types of pneumococci in the lungs in acute lobar pneumonia is shown by our statistics at the Hospital of The Rockefeller Institute, where among about 700 cases of lobar pneumonia Type I pneumococci were present in 35 per cent, Type II in 30 per cent, Type III in 10 per cent, and pneumococci of

## CHART I.

*Bacteria Present in Pneumonia.*

	(?) Pneumococcus, Type I.	} Typical Lobar Pneumonia.
	(?) " " II.	
	" " II, atypical.	
	" " III.	
	" " IV.	
Broncho-pneumonia.	}	
Lobular pneumonia.		
Interstitial broncho-pneumonia.		
Atypical forms with abscess formation, etc.		
	Bacterium pneumoniae (Friedländer's bacillus).	
	Bacillus influenzae.	
	Streptococcus hemolyticus.	
	(?) Streptococcus non-hemolyticus.	
	Staphylococcus aureus.	
	(?) Staphylococcus albus.	
	Other bacteria, as	
	Bacillus pestis, B. typhosus, etc.	

the fourth group in 25 per cent of the cases. We are justified, therefore, in considering acute lobar pneumonia as an acute infectious disease, or better as a group of infectious diseases with similar clinical manifestations and characteristic lung lesions, in which lesions pneumococci of Types I, II, III or IV are always present, and I have so indicated on the chart which I have arranged. (Chart I.)

When we leave this part of our subject, the conditions become much more complex and difficult to analyze. We now leave the discussion of the etiology of a specific disease in which there has been shown to be harmony between the physiologic reactions on the part of the body, the pathological anatomy and the bacteria present,

and we now have to discuss the relation of various bacteria not to a disease primarily but to pathological lesions. I cannot here take the time to discuss the various forms of anatomical changes in inflammation of the lung. I have indicated certain of the terms employed on the chart. In general, these lesions seem to be less specific in character, that is, to bear less relation to the bacteria present, than does the lesion spoken of as lobar.

The fact that pneumococci are not only found in the lung lesions of lobar pneumonia, but may be present in the more non-specific lung inflammations, brings a certain amount of confusion into the subject. It has long been known that pneumococci may be associated with the secondary broncho-pneumonia of children and with so-called terminal broncho-pneumonia. With the appearance during the past few years of apparently primary broncho-pneumonia in adults, it has also been found that pneumococci may be the only organisms present in the lesions. In few cases so far studied, however, have the pneumococci present been of the more specific parasitic Types I and II. Personally, I have not seen these organisms exclusively associated with these lesions, but for the present the matter must remain undecided.

So far as Friedländer's bacillus is concerned, its exact relation to pneumonia is not certain. This is not a single species but is a group of organisms, members of which are not infrequently found in the normal mouth and accessory sinuses. First described as the chief bacterium concerned in lobar pneumonia, it was soon found that it occurred relatively seldom in lung lesions. In many instances its presence in the inflamed lung is manifestly simply due to its growth in a lung already invaded by certain other micro-organisms. In certain cases, however, study has indicated strongly that it was the only bacterium concerned. For instance, in one case occurring in the Hospital of The Rockefeller Institute, this organism was isolated from material obtained from the lung on puncture on the first day, and in another case from the circulating blood on the fourth day.

Nevertheless, the number of cases of pneumonia associated with this organism must be relatively few. Among 844 cases at the Hospital of The Rockefeller Institute, in only three did this organism seem to be the only or important one concerned. Both the lesions



and the clinical features in this infection differ somewhat from those of typical lobar pneumonia.

Staphylococcus infection of the lung probably occurs with greater frequency than has been recognized in the past, and our experience strongly suggests that in certain cases of pulmonary infection staphylococcus aureus may be the only bacterium concerned. In the winter of 1914-15, I saw two cases and in 1915-16 one case in which the relation of this organism to primary lung infection seemed quite definite. In one case, that of a schoolgirl, aged 14, previously perfectly well, there occurred a sudden onset of illness, with very early signs of lung involvement, peculiar sputum, which we have come to associate with this form of infection, containing staphylococci, and on the fourth day staphylococci were cultivated from material obtained by lung puncture and from the blood. At autopsy the infection seemed to be primarily pulmonary. In these cases there is a great tendency to necrosis of lung tissue with the formation of multiple abscess. As to the relation of these bacteria to the pneumonia of influenza during the past winter I shall speak later.

The association of streptococcus and *B. influenzae* with the lung lesions of broncho-pneumonia has long been recognized. It must be remembered, however, that in the past broncho-pneumonia occurring in a previously healthy adult has been comparatively rare. In 549 cases of apparently primary pneumonia studied by me before 1917, in six cases *B. influenzae*, and in seven cases streptococci were the only organisms that could be isolated and these organisms seemed to be the only ones related to the disease processes in the respective groups of cases. These figures must be accepted with some reservation, however, for in no case was the demonstration of the primary and independent relationship absolutely convincing.

It will be noted that in the chart and in my remarks I have confined myself solely to the discussion of the bacteria present in pneumonia and have delayed my discussion of their etiologic significance until the present.

What is meant by the etiology of a disease, as I understand it, is the event or train of events that disturbs that orderly physiologic balance in an individual that we speak of as health. When we consider the etiology of pneumonia we want to know just why in an

individual the orderly course of events is altered, and to know this we must also know why another individual under exactly the same circumstances remains healthy. In the case of the specific infectious diseases the growth of the specific micro-organism within or on the surface of the body is an essential factor. The disease cannot occur without the presence of the specific micro-organism. In certain instances its presence seems to be the only factor, but with natural infection, this is very rare. A single plague bacillus rubbed into the skin of rats may invariably result in infection. But in the natural occurrence of plague in human beings, the greatest amount of variation in the occurrence and severity of the disease exists. At one time the same bacteria may be responsible for an epidemic of the pneumonic type of plague and at another of the bubonic form. At one time and place the disease may occur with the greatest virulence, at another it is mild. In one place practically all individuals are attacked, in another very few. It is evident, therefore, that even in plague, one of the most specific and contagious diseases, other factors besides the presence of the organisms are concerned in etiology.

In lobar pneumonia it was formerly held that the presence of the pneumococcus was but the culminating event in a series of phenomena leading up to the onset of the disease. As long as all pneumococci were held to be identical, we were forced to that view, for pneumococci are known to be frequent inhabitants of the normal mouth, and it was generally believed that infection was autogenous, that is, the individual became infected with the organism already present in his mouth because other circumstances, unrelated to the organism, made that possible. However, it has been demonstrated that pneumococci of Types I and II are not ordinarily present in normal mouths but only in the mouths of those sick of the disease and occasionally in the mouths of persons closely in contact with the sick. The importance of mere presence of the organisms, therefore, as far as pneumonia due to pneumococci of Types I and II is concerned, becomes greater. This point of view has recently been greatly supported by the work of Blake and Cecil. They have been able to produce typical lobar pneumonia in monkeys by the simple injection into the trachea of very small amounts of cultures of pneumococcus Type I, even as little as 0.001 cc., inserting the needle between the

tracheal cartilages. Since in the previous production of pneumonia in animals marked alterations in the conditions in the lungs had been produced by the simultaneous injection of considerable amounts of fluid directly into the bronchi, the significance of this demonstration is considerable. It is not conceivable, however, that even in this type of pneumonia in man the arrival of organisms of this type in the lungs is the only factor concerned.

## CHART II.

### *Factors Concerned in Occurrence of Pneumonia.*

1. Factors relating to the bacteria.
  - (a) Variations in virulence.
  - (b) Size of dosage.
2. Factors relating to the individual.
  - (a) Natural immunity.
  - (b) Acquired immunity.
  - (c) Change in local resistance.  
Foreign bodies, chest injuries, etc.
  - (d) Variations in general or local resistance due to  
Age.  
Primary infections—coryza, influenza, measles, etc.  
Hunger, fatigue, exposure, etc.  
Alcohol.  
Ether.
3. Factors affecting transmission of bacteria.
  - (a) Crowding.
  - (b) Habits of individuals.
  - (c) Travel.
4. Factors with unknown modes of action.
  - (a) Temperature.
  - (b) Altitude.
  - (d) Dust.

In the second chart I have made an outline of certain other factors concerning which we have some knowledge. There may be and probably are many additional factors of which at present we are ignorant. (Chart II.)

I have only time to draw your attention to a few of these factors so far as they relate to lobar pneumonia.

In the first place, animal experimentation has shown that in this whole group of bacteria concerned in pulmonary infections, the properties on which *virulence* depend are extremely labile. For instance, pneumococci may by animal passage be rendered extremely virulent for mice, but on artificial cultivation they may lose this property just as rapidly. A very striking fact, however, is that in general (though this is not always true) pneumococci of Types I and II, the specific types, tend to maintain their virulence better than those of Type IV. This may explain why these types have come to be so exclusively associated with disease. It is significant in this connection that in the epidemics of lobar pneumonia so far carefully studied, pneumococci of these two fixed types have been the ones concerned.

We have little knowledge concerning the *natural immunity* of man to pneumococci, though reasoning from analogy it is probable that, in general, man is relatively little susceptible. This means that under ordinary circumstances few persons would acquire the disease, even though all were inoculated. It is a striking fact that both in man and animals there exists a considerable degree of individual immunity to natural infection with the micro-organisms of most of the diseases from which they suffer. This is best seen in animals, where artificial measures of prevention obscure the features less than is the case with man.

Pleuro-pneumonia of cattle is a very contagious disease, yet it is generally stated that when a herd, in which acquired immunity can be ruled out, becomes infected, practically never do all the cattle suffer from the disease; always a third or a fourth of them escape.

In a common disease like pneumonia *acquired immunity* must play a large rôle in determining whether or not one suffers from the disease. The importance of this factor has been assumed, probably quite correctly, in explaining the great incidence of pneumonia among individuals brought into intimate contact with new individuals in a new environment.

It is well known that pneumococci may induce mild local infections as well as the severe general disease, lobar pneumonia. Therefore, in time, most persons may acquire some degree of immunity to the pneumococci common in their environment. Let these persons, how-



ever, be brought into contact with an entirely new group of people, who carry other varieties of pneumococci. Possessing no acquired immunity to these newly encountered strains, these persons may suffer severely from pneumonia. This has been held to explain the great frequency of occurrence of pneumonia among the native miners on the Rand in South Africa, as long as new natives were constantly being imported. Since this practice has been stopped and the population rendered fairly permanent, a great lessening of incidence of pneumonia has occurred. The same thing was true in Panama. At first, when new native laborers were being imported, the disease prevailed to a very great extent. Later it disappeared. Vaughn has shown that the incidence of disease among troops from the south, most of them from rural districts, was largely in excess of that among the northern soldiers, many of whom were from cities and therefore had been in contact with all sorts of disease. The explanation of increased incidence of measles among the southern country boys is obvious, but probably the same factor, namely, the lack of acquired immunity, is also important in explaining their increased susceptibility to pneumonia, though other factors, such as those mentioned under 3, may also be of significance.

Time does not permit me to discuss further the other factors concerned in the etiology of acute lobar pneumonia, though many important and interesting facts have lately been discovered which have a direct bearing on this question. The point which I wish to impress is that even in the most characteristic and specific of the acute lung infections, it is not sufficient nor correct to say that it is caused by pneumococcus. The presence of the bacteria is but one of the many factors concerned in the etiology.

While this is true of lobar pneumonia, it is still more true of the less specific and typical forms of disease associated with acute pulmonary infections. Referring to the points which I have noted on the chart, I should like to discuss briefly the atypical pneumonia which has been so prevalent during the past two years. In November and December, 1917, among the soldiers suffering from pneumonia who were admitted to the Hospital of The Rockefeller Institute, there were five who were found to be suffering from atypical pneumonia. Studies of the sputum and cultures from the lungs indicated

that the infectious agents concerned in four of these cases were hemolytic streptococci, and in one case, *Staphylococcus aureus*. None of these cases had lately suffered from measles. Since in all our previous experience we had seen very few similar cases, the occurrence of all these cases in two months aroused our very great interest. At the same time there began to be reported at the Surgeon General's Office a very great prevalence of pneumonia at the various camps. In many of the camps measles was also prevailing and many of the cases of pneumonia occurred coincidentally with or followed this disease. In February, 1918, I was made a member of a commission sent by the Surgeon General to Fort Sam Houston, Texas, to investigate the pneumonia present there. Report of this study has been published, and today I only desire to refer to certain of our observations in relation to etiology. We found many cases of typical lobar pneumonia, but in addition very many cases with atypical pneumonia, and the bacteria found in these cases were, with great constancy, hemolytic streptococci. In many of these cases the pneumonia occurred with or followed measles; in others the streptococcus infection apparently complicated or followed acute lobar pneumonia. In some instances, however, the streptococcus pulmonary infection apparently started independently of any other disease. The lesions in the lungs of these cases infected with *S. hemolyticus* showed certain characteristic features. The clinical features also differed from those of acute lobar pneumonia, and empyema was an extremely frequent complication. Reports of similar findings in other camps were reported within a short time. During the winter soldiers were constantly admitted to the Hospital of The Rockefeller Institute from nearby camps, mostly from Camp Mills. Each month there appeared one or two of the streptococcus cases until March, when 31 of these cases were admitted. During April there were 2 cases and during May, 3, and none during the remainder of the summer. Altogether during the winter there were 43 such cases admitted to the Hospital of The Rockefeller Institute, 10 of these being civilians. We made every effort not to admit any patients suffering, or convalescent, from measles and as no cases of measles developed in the wards through contact with these patients, it is evident that measles was not an important factor in our cases. In certain instances

evidence was obtained that a primary pneumococcus lobar pneumonia had been originally present. In other cases the evidence was strongly against this supposition. From my own experience, therefore, and from that of other observers, we may conclude that during the winter of 1917-18 there occurred in the camps, and to a lesser extent among civilians, a very large number of cases of infection of the lungs with *Streptococcus hemolyticus*, in certain places this infection taking on an epidemic character. The explanation of the greatly increased frequency of occurrence of this form of infection is, of course, not entirely clear, but we have certain evidence which strongly indicates the following course of events. In the first place, measles became very prevalent among the soldiers, and this disease undoubtedly lowers resistance to infection of the lungs with streptococci (2d). Crowding, especially of the measles cases, facilitated the transfer of the infection (3a). Rapid serial transfer of any infectious agent through a given host is known to be the best way to increase its virulence for that host (1a). The men were allowed to leave the wards while still carriers of the virulent bacteria and many of them indulging in improper habits (3b) and being crowded (3a) with other men in barracks, the latter, suffering unaccustomed fatigue, exposure, etc. (2d), rapidly became infected. The organism finally so increased in virulence apparently (1a) that their mere presence in the trachea of otherwise healthy but susceptible individuals might be followed by disease. There may have been other factors of which we have no knowledge, and it is impossible to estimate the exact relative value of those I have mentioned. We have considerable evidence, however, which justifies us in assuming the vicious circle I have described.

Under these circumstances it is not sufficient to say that the etiologic agent was *S. hemolyticus*. Many other factors were concerned and the etiology in the individual case, as well as the explanation of the epidemic, was complex.

The difficulty of offering any adequate explanation for the pneumonia occurring during the past winter is still more difficult, and I am only going to offer a few suggestions indicating that possibly the matter is not quite so simple as some would have it, and that in the final solution, more than one factor must be taken into consideration.



It is impossible at the present time to discuss adequately the etiology of influenza. The rapid spread of the epidemic can hardly be explained except on the assumption that the essential factor was a single infectious agent spreading among a susceptible population. The only two suggestions so far made as to the nature of this agent which are supported by any reliable evidence are, first, that it is an ultra-microscopic organism or a filterable virus, and second, that it is *B. influenzae*. Some evidence in favor of the first supposition has already been presented by Nicolle and Lebailly, Leschke, v. Angerer, Gibson and others. The evidence so far presented, however, cannot be considered convincing.

In regard to the second view, the actual proof of the primary relationship of *B. influenzae* is also lacking, as in a considerable number of cases this organism has not been demonstrated, and so far it has not been possible to reproduce the disease in animals. Whatever may be the nature of the primary infectious agent, however, the serious character of the disease is undoubtedly related to the pneumonia which may occur. That the disease itself is not necessarily severe is known from the reports of the epidemic as it occurred in the previous year in Europe, and from the history of earlier epidemics. It becomes severe, however, when pneumonia arises. In all the cases with pneumonia which have come to autopsy organisms of one or more of the following species have been present: *Bacillus influenzae*, pneumococcus (usually Type IV), streptococcus or staphylococcus. It seems likely that they have some relation to the disease process, especially since in some cases cultures were obtained by lung puncture very early in the disease. In a considerable number of the cases in certain series, especially in the cases studied by Wolbach, even in those instances in which other organisms were present in the lung lesions, *B. influenzae* was isolated as well. Wolbach from his pathological study thinks that in the pneumonia of influenza "we are dealing with a distinctive pathology," and it is evident that he is strongly of the opinion that this lesion is specifically related to the presence of *B. influenzae*. He does not disregard secondary infections by the other organisms I have mentioned, however, and thinks their growth in the lungs may modify the pathological picture and, I assume, the clinical picture as well. On the other



hand, other good observers, including MacCallum, have not been able to isolate influenza bacilli from many lungs from cases of influenza showing lesions of pneumonia. MacCallum believes that the conditions are exactly analogous to those in measles, and that some other virus so lowers resistance that secondary infection with *B. influenzae*, or with one of the other organisms which I have mentioned, may occur, this infection being followed by pneumonia, the lesions showing distinctive features, however, depending upon the variety of the secondary infecting organisms, but modified to some extent by the greatly lowered resistance, which allows very large numbers of the bacteria to grow.

My own observations on the pathology of the lung lesions do not permit me to offer any important additional evidence. My clinical experience, however, leads me to believe that lung lesions have been present, in New York, at least, in a larger proportion of the cases of the present epidemic than is generally considered to be the case. In most cases the lung lesion seemed to be a progressive one, the exact time of its onset being impossible to determine. In all of our cases coming to autopsy, staphylococci, streptococci or pneumococci were cultivated and in most cases *B. influenzae* as well, the latter, however, never alone. The relative frequency of staphylococcus infections in our cases during this epidemic should be mentioned. In 11 cases the association of this organism was demonstrated either by lung puncture, by culture from empyemal fluid or by cultures at autopsy. Chickering and Park have also drawn attention to this type of infection occurring in the cases at Camp Jackson. Of the 385 deaths occurring among a series of 1,409 cases of pneumonia in influenza, 153 were found to be associated with the presence of *Staphylococcus aureus* in the lungs. Whatever may be the organism primarily concerned in influenza, therefore, it seems not improbable that the high mortality in the late epidemic is largely to be ascribed to infection with one or other of these four micro-organisms. In view of the occurrences of the preceding winter, 1917-18, which I have described, one cannot help believing that the events of that winter played an important part in the present epidemic of influenza, and that the same factors to which I drew attention in considering that epidemic should not be overlooked in our consideration of the present one.

It is not unlikely that the high mortality of the past winter was related to the wide distribution of organisms of high virulence, to the conditions favoring low resistance on the part of individuals, especially soldiers, and to the existence of the unusual circumstances (war, increased travel) which favored the spread of pathogenic bacteria. How much of a rôle these same factors played in the occurrence of influenza must be left for the present unanswered. If, as is still possible, the specific agent in influenza is *B. influenzae*, these factors probably played a most important part.

The chief point which I have endeavored to present in this discussion is that in considering the etiology of pneumonia we cannot consider only the pathogenic micro-organisms. Many other factors must also be taken into account. In few cases of pneumonia, possibly in none, does the disease occur simply because the pathogenic micro-organism gains access to the lung. A person suffers from pneumonia as he does from other accidents, through a combination of circumstances. Moreover, epidemics also arise when a proper combination of circumstances occurs. By focusing attention on one factor alone the explanation sought for may not be found.



## THE DETERMINATION OF $\beta$ -HYDROXYBUTYRIC ACID, ACETOACETIC ACID, AND ACETONE IN BLOOD.

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In the determination of acetone bodies in the blood as described by us,<sup>1</sup> we neglected to state that the precipitate should be filtered soon after the period of boiling is ended. If the mixture is allowed to cool and stand for some hours, several mg. of flocculent precipitate of indefinite origin may form and cause a plus error of appreciable magnitude in the results.

<sup>1</sup> Van Slyke, D. D., and Fitz, R., Studies of acidosis. VIII. The determination of  $\beta$ -hydroxybutyric acid, acetoacetic acid, and acetone in blood, *J. Biol. Chem.*, 1917, xxxii, 495.





## STUDIES ON THE BIOLOGY OF STREPTOCOCCUS.

### I. ANTIGENIC RELATIONSHIPS BETWEEN STRAINS OF STREPTOCOCCUS HÆMOLYTICUS.

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(Received for publication, June 1, 1919.)

The importance of the problem of the systematic classification of bacteria for the proper understanding and control of infectious diseases is becoming increasingly evident. Such study is necessary not only in the elucidation of the biological relationship existing between varieties of the same species of bacterium, but is also essential to the working out of epidemiological problems and to the development of knowledge useful in the effort to control infectious diseases by means of specific therapeutic and prophylactic measures. Bacteria closely resembling those responsible for the pathological process in many acute infections have been found to be present and to live, apparently without harm to the host, on the mucous membranes of a large proportion of normal individuals. The resemblance of the pathogenic to the harmless variety of microorganism is frequently so close that in many instances tests of particular specificity are required to show the existing biological differences. In fact, the problem in etiology today is to determine not only the bacterial species causing a given disease, but in addition the number of varieties of the same species that are pathogenic, and whether common and important non-pathogenic varieties exist. The study is one of varying complexity, and methods suitable to one species do not give the desired information when applied to another.

The purpose of such studies may be broadly defined as an effort to relate fixed and determinable characteristics of bacteria to pathogenicity. Though fluctuating variations of bacteria probably occur, it seems not unlikely that in most diseases a sufficiently constant equilibrium has been attained to justify the usefulness of the effort. The

methods employed in these studies are numerous. In some species morphological and cultural characters give important though somewhat limited information, and in many the biochemical reactions are of great significance. The most serviceable method, however, for obtaining the particular kind of knowledge desired is the study of immunological relationships. For some as yet unexplained reason the latter specific reactions are very constant among the pathogenic varieties. In the following study of *Streptococcus hemolyticus* chief dependence has been placed on the knowledge obtained from the study of these immunity reactions.

In the various classifications of the streptococcus group as a whole that have been proposed, the custom has been to consider the strains that effect hemolysis of red blood cells as constituting a single or unit type (1). The validity of this assumption has been questioned and there has been much study and discussion of the probability of the existence of variations within this group, some evidence of which has been obtained from the study of sugar fermentations (2). Recent studies (3) indicate that certain broad lines of differentiation may be shown between hemolytic streptococci of human origin, and those of bovine origin whether found in milk or cheese. Hemolytic streptococci of the human type are usually found in association with some pathological process such as puerperal sepsis, septicemia, erysipelas, bronchopneumonia, or other conditions, and hemolytic activity is generally considered as an indication of pathogenicity. With the development of our knowledge (4), however, these organisms have been found with increasing frequency when no pathological lesion has been apparent. Investigators for many years have been interested in these strains, and discussion has centered about the unity or multiplicity of the group (5). The evidence in general favors the belief that hemolytic streptococci pathogenic for human beings comprise a single type. In the present paper it will be shown that by the use of properly controlled immune reactions differential characters between individual strains can be shown to exist.

During the winter of 1917-18 in the United States, there occurred in numerous localities a great increase in the incidence of a previously rather infrequent type of bronchopneumonia. The highest morbidity rate and earliest appearance of this disease were in military cantonments. In the spring of 1918, however, the type of infection under consideration was commonly observed in the civilian population. The disease first appeared as a secondary pneumonia following measles, but soon instances of apparently primary infection of the lungs were observed. Numerous studies of the bacteriology

of this condition have demonstrated that the infectious agent responsible for the lung lesion was in almost every instance *Streptococcus hemolyticus*. As a result of the widespread incidence of the disease the latter organism became extensively distributed and was frequently found as a secondary invader in acute lobar pneumonia, and as a common inhabitant of the normal throat.

The material used in the present study was obtained from the military establishments in the neighborhood of Fort Sam Houston, Texas. The sources of the individual strains were the throats of patients suffering from acute measles, the sputum of patients with bronchopneumonia both primary and secondary to measles, pathological material obtained from cases of bronchopneumonia and acute lobar pneumonia, and the throats of healthy individuals who had been directly or indirectly exposed to infection in a variety of ways.

All the strains of hemolytic streptococcus employed in this study possess the typical characteristics of the group. They are hardy organisms and grow readily in meat infusion broth and on blood agar slants. They survive for many months when grown for 18 hours in rabbit blood broth and subsequently placed at refrigerator temperature. In meat infusion broth the growth has been of two types—a granular sediment with clear supernatant fluid and a flocculent sediment with turbidity throughout the remainder of the tube. All the organisms are strongly Gram-positive, grow in chains of varying length, and are bile-insoluble. Capsule formation has not been observed. On plates two types of colonies are seen—a small, round, smooth colony and a moist ameboid colony with a slightly roughened surface. All the strains are actively hemolytic, showing a wide zone of hemolysis on the surface of rabbit blood agar plates; hemolysis is complete in 2 hours, when a 5 per cent suspension of washed rabbit blood cells suspended in salt solution is mixed with an equal quantity of a 24 hour broth culture. The power of the different strains to ferment the usual test substances for streptococcus has been studied. The majority fall in the group of *Streptococcus pyogenes* according to Holman's classification of streptococcus on a basis of sugar fermentations. None of the strains ferments inulin and about 20 per cent of these actively hemolytic strains possess the power of fermenting mannite. The latter characteristic will be shown to have an inter-



esting relationship to the immunological classification of these organisms.

The virulence of this group of hemolytic streptococci is low for the ordinary laboratory animals in comparison with such an organism, for instance, as pneumococcus. Doses of 1 cc. or more of a 24 hour broth culture administered intraperitoneally are required to kill guinea pigs and rabbits. Furthermore, repeated passages through these animals fail to bring about a considerable accession of virulence. The fatal dose for white rats and mice is smaller, usually in the neighborhood of 0.1 cc. of a broth culture. By continuous passage through rats and mice it has been possible to raise the virulence of a certain number of strains to a point where 0.001 cc. of broth culture is lethal for a rat in 24 hours and 0.00000001 cc. for a white mouse. On the other hand, many strains cannot be raised to this high degree of virulence even after the most persevering effort. Once the maximum of virulence is attained, this quality persists without renewed animal passages for an indefinite period of time.

The sources of the strains of *Streptococcus hæmolyticus* studied and some of their common characteristics are shown in Table I.

Although the finer differential classification of single species of bacteria by means of immune reactions is still in the earlier stages of development, enough evidence has been gathered to indicate that the more highly parasitic varieties of the species are more likely to consist of a limited number of unit types than are the less parasitic or the saprophytic members. In other words, unity of type seems to characterize the disease-producing microorganisms, whereas heterogeneity is more common among the non-pathogens. If this assumption is true it then becomes important to choose for purposes of classification the immune reactions which bring out most sharply the kind of differences sought, rather than a reaction which develops the basic relationship existing between all strains of the same species. For this purpose we regard the reactions of agglutination and protection as of superior usefulness to those of precipitation and complement fixation. The validity of any final classification arrived at depends, of course, upon the possibility of fitting accurately into such a classification a large number of strains freshly obtained from their natural environment.

TABLE I.

*Source and Common Characters of Strains of Streptococcus hemolyticus Studied.*

Strain No.	Source.	Clinical diagnosis.	Hemolysis.	Sugar fermentations.				
				Lactose.	Salicin.	Mannite.	Inulin.	Raffinose.
Type S 3.								
S 5	Autopsy (lung).	Bronchopneumonia following measles.	++++	+	+	-	-	-
S 29	Sputum.	“ “	++++	+	+	-	-	-
S 114	“	“ “	++++	+	+	-	-	-
S 118	Pleural fluid.	“ “	++++	+	+	-	-	-
S 124	Sputum.	“ “	++++	+	+	-	-	-
S 145	Throat.	“ “	++++	+	+	-	-	-
S 146	Chest fluid.	“ “	++++	+	+	-	-	-
S 149	Blood.	“ “	++++	+	+	-	-	-
S 151	Pleural fluid.	“ “	++++	+	+	-	-	-
S 2	Autopsy (blood).	Bronchopneumonia.	++++	+	+	-	-	-
S 3	“ (lung).	“	++++	+	+	-	-	-
S 14	“ “	“	++++	+	+	-	-	-
S 111	Chest fluid.	“	++++	+	+	-	-	-
S 53	Throat.	Measles.	++++	+	+	-	-	-
S 64	“	“	++++	+	+	-	-	-
S 154	“	“	++++	+	+	-	-	-
S 80	“	German measles.	++++	+	+	-	-	-
S 140	“	“ “	++++	+	+	-	-	-
S 11	Sputum	Lobar pneumonia (Type I).	++++	+	+	-	-	-
S 16	“	“ “	++++	+	+	-	-	-
S 31	Throat.	“ “	++++	+	+	-	-	-
S 44	“	“ “	++++	+	+	-	-	-
S 83	“	“ “	++++	+	+	-	-	-
S 95	“	“ “	++++	+	+	-	-	-
S 125	“	“ “	++++	+	+	-	-	-
S 144	“	“ “	++++	+	+	-	-	-
S 8	“	Pneumonia.	++++	+	+	-	-	-
S 131	“	“	++++	+	+	-	-	-
S 41	“	Incipient tuberculosis.	++++	+	+	-	-	-

TABLE I—*Continued.*

Strain No.	Source.	Clinical diagnosis.	Hemolysis.	Sugar fermentations.				
				Lactose.	Salicin.	Mannite.	Inulin.	Raffinose.
Type S 23.								
S 39	Autopsy (lung).	Bronchopneumonia following measles.	++++	+	+	-	-	-
S 78	Pleural fluid.	“ “	++++	+	+	-	-	-
S 101	“ “	“ “	++++	+	+	-	-	-
S 107	Sputum.	“ “	++++	+	+	-	-	-
S 27	Autopsy (blood).	Bronchopneumonia.	++++	+	+	-	-	-
S 67	Blood.	“	++++	+	+	-	-	-
S 120	Autopsy (blood).	“	++++	+	+	-	-	-
S 98	Throat.	Measles.	++++	+	+	-	-	-
S 116	“	“	++++	+	+	-	-	-
S 117	“	“	++++	+	+	-	-	-
S 9	“	Lobar pneumonia.	++++	+	+	-	-	-
S 23	“	“ “	++++	+	+	-	-	-
S 56	Autopsy (lung).	“ “	++++	+	+	-	-	-
S 65	Sputum.	“ “	++++	+	+	-	-	-
S 75	Throat.	“ “	++++	+	+	-	-	-
S 133	“	“ “	++++	+	+	-	-	-
S 104	“	Pneumonia.	++++	+	+	-	-	-
S 130	“	“	++++	+	+	-	-	-
S 46	“	Incipient tuberculosis.	++++	+	+	-	-	-
S 122	“	“ “	++++	+	+	-	-	-

TABLE I—Continued.

Strain No.	Source.	Clinical diagnosis.	Hemolysis.	Sugar fermentations.				
				Lactose.	Salicin.	Mannite.	Inulin.	Ralinose.
Type S 60.								
S 137	Pleural fluid.	Bronchopneumonia following measles.	++++	+	+	+	-	-
S 6	Autopsy (lung).	Bronchopneumonia.	++++	+	+	+	-	-
S 35	Pleural fluid.	“	++++	+	+	+	-	-
S 55	Sputum.	“	++++	+	+	+	-	-
S 10	Throat.	Measles.	++++	+	+	+	-	-
S 37	“	“	++++	+	+	+	-	-
S 43	“	“	++++	+	+	+	-	-
S 60	“	“	++++	+	+	+	-	-
S 66	“	“	++++	+	+	+	-	-
S 71	“	“	++++	+	+	+	-	-
S 86	“	“	++++	+	+	+	-	-
S 88	“	“	++++	+	+	+	-	-
S 89	“	“	++++	+	+	+	-	-
S 100	“	“	++++	+	+	+	-	-
S 109	“	“	++++	+	+	+	-	-
S 123	“	“	++++	+	+	+	-	-
S 127	“	“	++++	+	+	+	-	-
S 128	“	“	++++	+	+	+	-	-
S 4	“	German measles.	++++	+	+	+	-	-
S 19	“	Lobar pneumonia.	++++	+	+	+	-	-
S 62	“	“ “	++++	+	+	+	-	-
S 72	“	“ “	++++	+	+	+	-	-
S 21	“	Incipient tuberculosis.	++++	+	+	+	-	-
S 141	“	“ “	++++	+	+	+	-	-
S 150	“	“ “	++++	+	+	+	-	-
S 267	Foot.	Cellulitis.	++++	+	+	+	-	-
S 269	Blood.	Erysipelas.	++++	+	+	+	-	-



TABLE I—*Continued.*

Strain No.	Source.	Clinical diagnosis.	Hemolysis.	Sugar fermentations.				
				Lactose.	Salicin.	Mannite.	Inulin.	Rafinose.
Type S 84.								
S 1	Autopsy (lung).	Bronchopneumonia following measles.	++++	+	+	-	-	-
S 20	“ (blood).	“ “	++++	+	+	-	-	-
S 110	Sputum.	“ “	++++	+	+	-	-	-
S 138	“	“ “	++++	+	+	-	-	-
S 50	Pleural fluid.	Bronchopneumonia.	++++	+	+	-	-	-
S 84	“ “	“	++++	+	+	-	-	-
S 139	Throat	“	++++	+	+	-	-	-
S 115	“	German measles.	++++	+	+	-	-	-
S 15	“	Lobar pneumonia.	++++	+	+	-	-	-
Unclassified.								
S 32	Autopsy (lung).	Bronchopneumonia following measles.	++++	+	+	-	-	-
S 59	“ (blood).	“ “	++++	+	+	-	-	-
S 93	Sputum.	“ “	++++	+	+	-	-	-
S 97	Pleural fluid.	“ “	++++	+	+	-	-	-
S 136	Autopsy (lung).	“ “	++++	+	+	-	-	-
S 142	Sputum.	“ “	++++	+	+	-	-	-
S 49	“	Bronchopneumonia following German measles.	++++	+	+	-	-	-
S 24	Autopsy (lung).	Bronchopneumonia.	++++	+	+	-	-	-
S 18	Throat.	Measles.	++++	+	+	-	-	-
S 26	“	“	++++	+	+	-	-	-
S 42	“	“	++++	+	+	-	-	-
S 51	“	“	++	+	+	-	-	+
S 63	“	“	++++	=	+	-	-	-
S 96	“	“	++++	+	+	-	-	-
S 102	“	“	++++	+	+	-	-	-
S 106	“	“	++++	+	+	-	-	-
S 108	“	“	++++	=	+	-	-	-
S 148	“	“	++++	+	+	-	-	-

TABLE I—*Concluded.*

Strain No.	Source.	Clinical diagnosis.	Hemolysis.	Sugar fermentations.				
				Lactose.	Salicin.	Mannite.	Inulin.	Rafinose.
Unclassified— <i>Concluded.</i>								
S 17	Throat.	German measles.	+	—	—	+	—	—
S 47	“	“ “	++++	+	—	—	—	—
S 48	“	“ “	++++	+	+	—	—	—
S 54	Sputum.	“ “	++++	+	+	—	—	—
S 68	Throat.	“ “	++++	+	+	—	—	—
S 69	Autopsy (lung).	Lobar pneumonia.	++++	+	+	—	—	—
S 87	Sputum.	“ “	++++	+	+	—	—	—
S 90	Throat.	“ “	++++	+	+	—	—	—
S 99	Sputum.	“ “	++++	+	+	—	—	—
S 34	Throat.	Incipient tuberculosis.	++++	+	+	+	—	+
S 36	“	“ “	++++	+	+	—	—	—
S 121	“	“ “	++++	+	—	—	—	—
S 129	“	“ “	++++	+	+	—	—	—
S 155	“	“ “	++++	+	+	—	—	—
S 264	Blood.	Osteomyelitis.	++++	+	+	—	—	—
S 271	“	Septicemia.	++++	+	+	—	—	—
S 272	Pus.	Abscess (measles).	++++	+	+	—	—	—
S 273	“	Scarlet fever.	++++	+	+	—	—	—
S 276	“	Pelvic abscess.	++++	+	+	—	—	—
S 277	“ (abdomen).	Peritonitis.	++++	+	+	—	—	—
S 286	Pleural fluid.	Pneumonia.	++++	+	+	—	—	—
S 288	Sputum.	“	++++	+	+	—	—	—

*The Reaction of Agglutination.*

Specific agglutination has been found to be one of the most serviceable immune reactions for purposes of the biological classification of bacteria. In the typhoid and pneumococcus groups, for instance, it serves to distinguish clearly the different varieties from one another. It is likewise applicable to the classification of many other microorganisms. Efforts to classify the streptococci by means of this reaction apparently have not illuminated materially the relationship of one strain to another, nor have they shown a definite relationship between certain strains and a particular pathological process. A number of explanations of this fact may be proposed. In many instances no attention has been paid to the broader groupings of streptococci as determined by hemotoxin production and the fermentation of test sugars. Also streptococcus most frequently acts as a secondary invader in the production of disease, and it is probably an unwarranted assumption to suppose that type specificity is closely related to the character of the pathological process. One of the chief obstacles to the successful carrying out of the agglutination reaction has been the tendency of all types of streptococcus to undergo spontaneous granulation, and when used for tests to exhibit the phenomenon of non-specific agglutination. As a result of this, the reactions must usually be read against a more or less granular background, making it difficult, if not impossible, to distinguish between the non-specific and the specific influences. The tendency to spontaneous clumping is occasioned by several factors, only a few of which are understood. For instance, a homogeneous suspension of a granular streptococcus can easily be prepared by washing the organism several times with distilled water, and then resuspending in the same medium. The suspension will remain homogeneous for an indefinite period of time. If sodium chloride in concentrations above 0.06 per cent is added to the suspension, granulation immediately ensues. Many other salts act in the same manner. Substances antagonistic to this salt action may be added to the medium and function even to the extent of suspending the participation of the salt in the immune reaction, so that specific agglutination may be completely inhibited. Fortunately intermediate combinations can be found in which most

strains remain diffuse and in which the salt is still able to fulfill its part in the immune reaction. The most useful substance of this kind is ordinary meat infusion broth to which 1 per cent peptone has been added.<sup>1</sup> In addition, if streptococci are exposed to too great acidity the tendency to granulation is increased. In order to avoid this, the reaction of the medium may be so adjusted and such quantities of balanced phosphate solutions added that during growth an acidity greater than pH 7.1 is not attained. Certain other undetermined factors cause granulation, which may be defined as a general unsuitability of the medium for growth, and these can be eliminated only by experimenting with different preparations.

### *Technique.*

The immune sera used in the agglutination and protection tests were obtained by the immunization of rabbits, sheep, and dogs. The animals were inoculated intravenously with repeated doses of heat-killed organisms, and in most instances a certain number of doses of living organisms was given. The employment for immunization of freshly isolated unpassed human strains, or the use of the same strains after a series of animal passages, does not alter in any recognizable way the specific qualities of the serum. The agglutinin and protective titer of the sera has remained undiminished for many months after the time of bleeding.

Great care is taken in the preparation of the organisms to be used in the agglutination reaction. The broth is made from carefully selected meat, and instead of the usual sodium chloride a sufficient quantity of a balanced phosphate mixture is added to give the required salt concentration and to adjust the hydrogen ion concentration at a pH of 7.4. When *Streptococcus hemolyticus* is grown for 24 hours in a medium to which no sugar has been added, it does not develop an acidity greater than pH 7.2, which is just above the point at which the tendency to granulation appears. The organisms are removed from the culture medium by centrifugalization and are

<sup>1</sup> The authors are greatly indebted to Dr. Charles Krumwiede, Jr., of the Research Laboratories of the Department of Health of the City of New York, for many helpful suggestions in this technique.





TABLE III.

*Test of Cross-Agglutination Reactions of Antistreptococcus Serum, Type S 3, with Strains of Streptococcus hemolyticus of Other Types and with Unclassified Strains.*

[illegible]

allowed to continue longer, non-specific granulation occurs. If clumping develops in the broth controls or in more than the first two or three dilutions of normal serum, the reaction should be regarded as unsatisfactory and discarded. By the use of this technique, it has been possible to carry out reliable agglutination tests of various strains of *Streptococcus hæmolyticus* and to show that constant type rela-

TABLE IV.

*Power of Antistreptococcus Serum, Type S 23, to Agglutinate Ten Representative Strains of the Same Type.*

Strain No.	Serum.	Dilution.									
		1: 20	1: 40	1: 80	1: 160	1: 320	1: 640	1: 1,280	1: 2,560	1: 5,120	Broth.
S 23	Type S 23	++	++	++	++	++	++	+	-	-	-
	Normal.	-	-	-	-	-	-	-	-	-	-
S 107	Type S 23	++	++	++	++	++	++	+ ±	+	±	-
	Normal.	-	-	-	-	-	-	-	-	-	-
S 27	Type S 23	+	++	++	++	+ ±	+	±	-	-	-
	Normal.	-	-	-	-	-	-	-	-	-	-
S 39	Type S 23	+	+	+ ±	++	+ ±	+	±	-	-	-
	Normal.	-	-	-	-	-	-	-	-	-	-
S 56	Type S 23	+	+	+ ±	+ ±	+ ±	+	±	-	-	-
	Normal.	-	-	-	-	-	-	-	-	-	-
S 67	Type S 23	+	+	+ ±	+ ±	+	+	±	-	-	-
	Normal.	-	-	-	-	-	-	-	-	-	-
S 98	Type S 23	+ ±	++	++	++	++	+ ±	+ ±	+	-	-
	Normal.	-	-	-	-	-	-	-	-	-	-
S 101	Type S 23	+ ±	++	++	++	++	+ ±	+ ±	+	±	-
	Normal.	-	-	-	-	-	-	-	-	-	-
S 104	Type S 23	+	++	++	++	++	++	+ ±	+	-	-
	Normal.	-	-	-	-	-	-	-	-	-	-
S 130	Type S 23	+ ±	+ ±	+ ±	++	++	+ ±	+	+	+	-
	Normal.	-	-	-	-	-	-	-	-	-	-

tionships exist, and that the types are sharply differentiated from one another. The results of the application of the method to strains of streptococcus described above are shown in Tables II to IX.

In these tables are presented the agglutination reactions of a certain proportion of the total number of strains of *Streptococcus hæmolyticus* tested. An analysis of the results shows that in the collection of

TABLE V.

*Test of Cross-Agglutination Reactions of Antistreptococcus Serum, Type S 23, with Strains of Streptococcus hæmolyticus of Other Types and with Unclassified Strains.*

[illegible]



organisms studied it has been possible to detect four different types of *Streptococcus hæmolyticus*. These types have been noted as Types S 3, S 23, S 60, and S 84, from the number of the chosen representative. In addition to the type strains, there remains a residue of unclassified organisms. The summary for the total number of strains studied is given in Table X.

TABLE VI.

*Power of Antistreptococcus Serum, Type S 60, to Agglutinate Ten Representative Strains of the Same Type.*

Strain No.	Serum.	Dilution.									
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	Broth.
S 60	Type S 60	++	++	++		++		++	++	+	—
	Normal.	—	—	—		—		—	—	—	—
S 269	Type S 60	++	++	++	++	+	+	+	—		—
	Normal.	+	—	—	—	—	—	—	—		—
S 267	Type S 60	++	++	++	++	++	++	+	+		—
	Normal.	+	—	—	—	—	—	—	—		—
S 55	Type S 60	++	++	++		++		+	+	+	—
	Normal.	—	—	—		—		—	—	—	—
S 128	Type S 60	++	++	++		++		++	++	+	—
	Normal.	—	—	—		—		—	—	—	—
S 72	Type S 60	++	++		++		++	+	+	+	—
	Normal.	+	+		—		—	—	—	—	—
S 43	Type S 60	++	++		++		++	++	++	+	—
	Normal.	—	—		—		—	—	—	—	—
S 123	Type S 60	++	++		++		++	++	++	+	—
	Normal.	—	—		—		—	—	—	—	—
S 66	Type S 60	++	++		++		++	++	+	+	—
	Normal.	—	—		—		—	—	—	—	—
S 127	Type S 60	++	++		++		++	++	++	+	—
	Normal.	—	—		—		—	—	—	—	—

The total number of strains of *Streptococcus hæmolyticus* studied was 125. Of these, 85, or 68 per cent, are comprised in the types mentioned above, and 40, or 32 per cent, remain unclassified. Work with the unclassified strains is being continued and the indications are that a certain number of other types will be discovered. In fact, two new types have already been encountered, one comprising five

TABLE VII.

*Test of Cross-Agglutination Reactions of Antistreptococcus Serum, Type S 60, with Strains of Streptococcus hæmolyticus of Other Types and with Unclassified Strains.*

[illegible]

strains and another four strains, the immune reactions of which have not as yet been completed.

The antistreptococcus sera used in the agglutination reaction were obtained in the main by the immunization of sheep, a species of animal which yields a highly specific agglutinating serum for *Streptococcus hæmolyticus*. Agglutination occurred in all the type sera in dilutions of 1:1,000 or higher, with the exception of Type S 84, of which the

TABLE VIII.

*Power of Antistreptococcus Serum, Type S 84, to Agglutinate Eight Representative Strains of the Same Type.*

Strain No.	Serum.	Dilution.									
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	Broth.
S 84	Type S 84	+	±								—
	Normal.	—		—			—				
S 1	Type S 84	+	±	+	±	+	±				—
	Normal.	—	—	—	—	—	—				
S 50	Type S 84	+	±	+	±	+	±	+			—
	Normal.	—	—	—	—	—	—				
S 20	Type S 84	++	++	+	±	±	—	—			—
	Normal.	—	—	—	—	—	—				
S 15	Type S 84	+	±	±	±	+		—			—
	Normal.	—	—	—	—	—					
S 115	Type S 84	+	±	±	±	±	±				—
	Normal.	—	—	—	—	—	—				
S 139	Type S 84	+	±	±	±	±	+				—
	Normal.	—	—	—	—	—	—				
S 138	Type S 84	+	±	±	±	±	+				—
	Normal.	—	—	—	—	—	—				

agglutination titer has been consistently lower, usually not above 1:320. The agglutination titer of all the type sera for each strain of the same type has been approximately equal to the titer for the organism used for purposes of immunization. There has been strikingly little cross-agglutination between serum of one type and strains belonging to another. The same lack of crossing is observed among the unclassified strains with the few exceptions in which certain of these strains have shown some degree of agglutination in the type

TABLE IX.

*Test of Cross-Agglutination Reactions of Antistreptococcus Serum, Type S 84, with Strains of Streptococcus hæmolyticus of Other Types and with Unclassified Strains.*

[illegible]



sera, but not in sufficiently high dilutions to justify their inclusion within the types. These facts show that it is possible by a series of carefully conducted agglutination experiments to determine specific type relationships between strains of *Streptococcus hæmolyticus* and to show that the different types are immunologically distinct from one another. The clearness of the agglutination reactions presented is somewhat deceptive as to the ease and simplicity of the test. It must be remembered that *Streptococcus hæmolyticus* is notoriously

TABLE X.  
*Summary of Agglutination Reactions.*

Type of <i>S. hæmolyticus</i> .	No. of strains.	Per cent.
S 3.....	29	23.2
S 23.....	20	16.0
S 60.....	27	21.6
S 84.....	9	7.2
Unclassified.....	40	32.0
Total typed.....	85	68.0
“ strains studied.....	125	

variable in its reactions, and that very slight and indeterminable changes in technique frequently obliterate almost completely the specificity of the agglutination reaction. In addition, a considerable number of strains is invincibly granular under all conditions and cannot be used, and occasionally strains are encountered which may occupy intermediate positions, the exact understanding of which needs a technique for the conduction of absorption experiments.

### *The Reaction of Protection.*

Study of the power of antistreptococcus serum to protect animals against experimental infection with this organism has given rise to a number of different points of view, both regarding its action against strains from different sources, and against the same strain before and after animal passage, and also concerning the kind and the different

effect of varying antigens used in the process of immunization. For a full discussion of these matters the reader is referred to the general articles on streptococcus and to the more important papers dealing with these particular points (6). In this work they will only be considered where they have a particular bearing upon the subject under investigation. Although in the present paper the classification of *Streptococcus hæmolyticus* by means of the agglutination reaction has been presented first, practically we have obtained our primary indications of the degree of antigenic differences between strains by means of the reaction of protection. Later each reaction has been used to confirm the information obtained by means of the other.

In the successful carrying out of protection experiments two points are of especial importance: first, the production of a serum of high potency; and second, the possibility of raising the virulence of the test strains of streptococcus to such a point that very minute doses of culture are sufficient to kill white mice in a limited period of time. We have been able to produce sera in the manner alluded to above of such potency that 0.5 cc. administered intraperitoneally is sufficient to protect a white mouse against 100,000 lethal doses of a highly virulent streptococcus. In order to produce such a serum many animals must be used, only a few of which may give the desired result. It has been possible to raise the virulence of many strains by continuous passage through white mice and rats to such a point that doses of from 0.000001 to 0.00000001 cc. of broth culture are sufficient to kill the former animals in from 24 to 48 hours. These seemingly difficult conditions must be attained in order that sufficiently long-range protective titers may be carried out to insure the reliability of the information obtained. Protection against one or two lethal doses of a series of strains of streptococcus by a monovalent serum is subject to so many interpretations that the evidence gained cannot be considered of much value in judging accurately the antigenic relationship of the different strains.

The technique observed in the protocols given below has been as follows: The potency of all sera has been titrated for the homologous strain of organism and only the sera which gave a wide range of protection have been used. For infection, virulent streptococci have been used which have been grown for approximately 18 hours in either

plain broth or ascites broth. In the inoculation of animals the technique advised by Neufeld (5) has been followed with only a minor variation. The test animals have been injected intraperitoneally with 0.5 cc. of serum 24 hours before the conduction of the experiment. Tentative trials have shown that if the serum is given simultaneously with the infecting dose, no protection results, and that to insure success the serum must be given at least 8 hours before infection. On the following day a series of virulence controls is inoculated intraperitoneally, and the serum animals are injected in the same manner with doses of cultures ranging from 0.001 to 0.00000001 cc. of broth culture. Animals surviving for a period of 5 days are considered to be adequately protected. By the use of this method, it has been possible to test the antigenic relationship of a considerable number of virulent strains of *Streptococcus hemolyticus*, and the results of these tests are set forth in the following protocols.

*Protocol 1.*

In this protocol is shown the titration of the serum of a sheep immunized against Strain S 23. The culture employed for infection was an 18 hour broth culture of No. S 23, which had received eighteen passages through white rats and mice. Each mouse had received 0.5 cc. of immune serum 24 hours previous to infection.

Virulence controls.		Protective power of Serum S 23.	
Dose of culture.	Result.	Dose of culture.	Result.
cc.		cc.	
		0.001	S.
		0.0001	D. in 4 days.
0.00001	D.* in 24 hrs.	0.000001	S.
0.000001	" " 24 "	0.0000001	"
0.0000001	" " 24 "	0.00000001	"

\*.In the tables D. indicates died, S. survived.

Protocol 2.

In this protocol is shown the protective power of Immune Serum S 3 for two virulent strains of the homologous type, for two strains of each of the heterologous types, and for two unclassified strains. The technique was the same as that in the previous protocol.

Streptococcus No.	Type of streptococcus.	Type of serum.	Dose of culture.				
			0.001 cc.	0.0001 cc.	0.000001 cc.	0.0000001 cc.	0.00000001 cc.
S 3.18 <sup>2*</sup>	S 3	S 3 No serum.	D. 14 hrs.	S.	S. D. 20 hrs.	S. D. 23 hrs.	
S 149.16 <sup>2</sup>	S 3	S 3 No serum.	S.	S. D. 36 hrs.	S. D. 24 hrs.		
S 39.3 <sup>3</sup>	S 23	S 3 No serum.	D. 24 hrs.	" 7 "	" 19 "	" 21 "	
S 67.7 <sup>2</sup>	S 23	S 3 No serum.	" 16 "	" 16 "	" 21 "		
			" 16 "	" 17 "	" 23 "	D. 19 hrs. " 21 "	
S 128.14 <sup>2</sup>	S 60	S 3 No serum.	" 8 "	D. 48 hrs. " 36 "	" 24 "	" 36 "	
S 60.10 <sup>2</sup>	S 60	S 3 No serum.	D. 24 hrs. " 24 "	" 36 "	" 20 "	" 24 "	
S 1.8 <sup>1</sup>	S 84	S 3 No serum.	" 36 "	S. D. 22 hrs. " 21 "	" 22 "	" 21 "	
S 84.17 <sup>2</sup>	S 84	S 3 No serum.	" 18 "	" 19 "	" 19 "	S. D. 21 hrs. " 19 "	D. 20 hrs. " 60 "
S 24.25 <sup>3</sup>	Unclassified.	S 3 No serum.	" 22 "	D. 19 hrs.	" 18 "	" 31 "	" 28 "
S 266.6 <sup>2</sup>	"	S 3 No serum.	D. 16 hrs.	D. 16 hrs. " 17 "	" 21 "	" 21 "	" 24 "

\* The integer indicates serial number of the culture, the decimal shows the number of animal passages, and the exponent the number of transplants since the last animal passage.



## Protocol 3.

In this protocol is shown the protective power of antistreptococcus serum, Type S 23, against two strains of the homologous type, against two strains of each of the heterologous types, and against two unclassified strains. The technique was the same as that employed in the previous protocols.

Streptococcus No.	Type of streptococcus.	Type of serum.	Dose of culture.				
			0.001 cc.	0.0001 cc.	0.00001 cc.	0.000001 cc.	
S 23.18 <sup>2</sup>	S 23	S 23 No serum.	D. 11 hrs.	S. D. 19 hrs.	S. D. 24 hrs.	S. D. 20 hrs.	
S 107.12 <sup>2</sup>	S 23	S 23 No serum.	S.	" 16 " " 24 "	S. D. 24 hrs.	S. D. 24 hrs.	
3.21 <sup>2</sup>	S 3	S 23 No serum.	D. 18 hrs.	" 18 " " 18 "	" 36 " " 36 "	" 64 " " 36 "	
S 80.7 <sup>2</sup>	S 3	S 23 No serum.	D. 18 hrs.	" 64 " S.	" 96 " " 36 "	S. D. 36 hrs.	
S 55.22 <sup>1</sup>	S 60	S 23 No serum.	D. 20 hrs.	D. 20 hrs. " 22 "	" 30 " " 20 "	" 30 " " 20 "	
S 60.10 <sup>1</sup>	S 60	S 23 No serum.	D. 20 hrs.	" 24 " " 20 "	S. D. 36 hrs.	S. D. 36 hrs.	
S 84.18 <sup>1</sup>	S 84	S 23 No serum.	D. 12 hrs.	" 18 " " 16 "	" 36 " " 20 "	D. 18 hrs. S.	
S 50.4 <sup>2</sup>	S 84	S 23 No serum.	D. 18 hrs.	" 18 " " 18 "	" 18 " " 36 "	D. 18 hrs. " 18 "	
S 276.31 <sup>2</sup>	Unclassified.	S 23 No serum.	D. 16 hrs.	" 66 " " 18 "	" 40 " " 40 "	" 40 " " 40 "	
S 24.31 <sup>2</sup>	"	S 23 No serum.	D. 16 hrs.	" 16 " " 16 "	D. 20 hrs.	D. 20 hrs.	

## Protocol 4.

In this protocol is shown the protective power of antistreptococcus serum, Type S 60, against two strains of homologous type, against two strains of each of the heterologous types, and against two unclassified strains. The technique was the same as that employed in the previous protocols.

Streptococcus No.	Type of streptococcus.	Type of serum.	Dose of culture.			
			0.001 cc.	0.0001 cc.	0.000001 cc.	0.0000001 cc.
S 60. 10 <sup>2</sup>	S 60	S 60 No serum.	D. 19 hrs.	S. D. 17 hrs.	S. D. 19 hrs.	
S 55. 22 <sup>2</sup>	S 60	S 60 No serum.	S.	S. D. 23 hrs.	S. D. 16 hrs.	
S 3. 22 <sup>2</sup>	S 3	S 60 No serum.	D. 18 hrs.	" 18 " " 18 "	" 30 " " 18 "	D. 24 hrs. " 18 "
S 80. 8 <sup>2</sup>	S 3	S 60 No serum.	D. 18 hrs.	" 18 " " 18 "	" 22 " " 18 "	" 24 " " 18 "
S 75. 6 <sup>1</sup>	S 23	S 60 No serum.	D. 18 hrs.	" 20 " " 17 "	D. 23 hrs.	•
S 23. 19 <sup>2</sup>	S 23	S 60 No serum.	D. 18 hrs.	" 18 " " 18 "	" 24 " " 60 "	D. 60 hrs. " 18 "
S 84. 12 <sup>1</sup>	S 84	S 60 No serum.	D. 18 hrs.	" 18 hrs. " 18 "	" 22 " " 20 "	
S 50. 5 <sup>1</sup>	S 84	S 60 No serum.	D. 18 hrs.	" 18 " " 18 "	" 18 " " 18 "	D. 18 hrs. " 18 "
S 24. 28 <sup>2</sup>	Unclassified.	S 60 No serum.	D. 17 hrs.	" 17 " " 17 "	" 24 " " 65 "	D. 31 hrs.
S 276. 31 <sup>2</sup>	"	S 60 No serum.	D. 9 hrs.	D. 20 hrs. " 23 "	" 41 " " 33 "	

## Protocol 5.

In this protocol is shown the protective power of antistreptococcus serum, Type S 84, against two strains of the homologous type, against two strains of each of the heterologous types, and against two unclassified strains. The technique was the same as that employed in previous protocols.

Streptococcus No.	Type of streptococcus.	Type of serum.	Dose of culture.				
			0.001 cc.	0.0001 cc.	0.000001 cc.	0.00000001 cc.	
S 84.16 <sup>1</sup>	S 84	S 84 No serum.	S. D. 16 hrs.	S. D. 26 hrs.	S. D. 54 hrs.		
S 20.5 <sup>1</sup>	S 84	S 84 No serum.	S. D. 30 hrs.	S. D. 55 hrs.	S. D. 55 hrs.		
S 3.16 <sup>1</sup>	S 3	S 84 No serum.	D. 17 hrs. " 17 "	" 20 " " 20 "	" 29 " " 29 "		
S 14.24 <sup>1</sup>	S 3	S 84 No serum.	D. 16 hrs. " 16 "	" 16 " " 16 "	" 16 " " 16 "		D. 16 hrs. " 40 "
S 23.16 <sup>1</sup>	S 23	S 84 No serum.	D. 18 hrs. S.	D. 33 hrs. " 22 "	" 32 " " 23 "		
S 107.7 <sup>1</sup>	S 23	S 84 No serum.	D. 18 hrs. D. 18 hrs.	" 24 " " 18 "	S. D. 18 hrs.		D. 24 hrs.
S 128.14 <sup>2</sup>	S 60	S 84 No serum.	D. 24 hrs. D. 24 hrs.	" 24 " " 24 "	" 24 " " 36 "		
S 60.10 <sup>2</sup>	S 60	S 84 No serum.	D. 18 hrs. " 18 "	" 18 " S.	" 21 " " 24 "		
S 152.5 <sup>1</sup>	Unclassified.	S 84 No serum.	D. 20 hrs. " 29 " " 16 "	D. 29 hrs. S.	" 76 " " 28 "		
S 266.5 <sup>2</sup>	"	S 84 No serum.	D. 15 hrs. " 34 " " 18 "	D. 23 hrs. " 38 "	" 45 " " 21 "		

Consideration of the above protocols shows that the type relationships manifest from the agglutination reactions have been substantiated by the evidence obtained from the protection tests. As a matter of fact, each reaction has been used to supplement the other, the first clue as to the position of an organism sometimes being obtained by protection and sometimes by agglutination. On the whole, we are inclined to place greater confidence in the reaction of protection than in that of agglutination, and would be slow to draw general conclusions concerning type specificity from agglutination alone with such a variable organism as streptococcus, unless the results of this test could be confirmed by some other specific reaction such as protection. The sera prepared, as is seen from the protocols, have afforded a high degree of protection to white mice against infection with organisms of the homologous type. Little or no protection results when serum of one type is employed against organisms of heterologous types. There are, of course, some exceptions to this general rule. Occasionally strains of *Streptococcus hemolyticus* are encountered against which all type sera afford a varying degree of protection, and sometimes a serum is obtained from one strain which will protect against an organism of another type, and when the reaction is reciprocally reversed no protection results. At present our knowledge is insufficient to discuss these intermediate reactions intelligently, and their elucidation must await further development of the technique. In all it has been possible to raise the virulence of 31 strains to a point where protection experiments could be performed. Of these, 7 belonged to Type S 3, 8 to Type S 23, 6 to Type S 60, 7 to Type S 84, and 3 to the unclassified group. In view of the difficulty of raising the virulence of the organisms it has been found advantageous to perform the reaction in two ways: first, to test a single monovalent serum against a number of strains; and second, to test a number of sera prepared from strains of the same type against a single virulent strain of that type. In Tables XI to XIV is shown a summary of the total number of protection experiments performed.



TABLE XI.

*Summary of the Protective Power of Antistreptococcus Serum, Type S 3, against Strains of the Homologous and Heterologous Types.*

Type of serum.	Strain of <i>S. hemolyticus</i> used for production of serum.	Strain of <i>S. hemolyticus</i> and type used for infection of mice.	Minimal lethal dose of <i>S. hemolyticus</i> used for infection.	Protective power of 0.5 cc. of serum.
			cc.	
S 3	S 3 (Rabbit 1).	S 3 (Type S 3).	0.00000001	S. 0.0001 cc.
S 3	S 3 ( " 1).	S 14 ( " S 3).	0.000001	" 0.001 "
S 3	S 3 (Dog 1).	S 3 ( " S 3).	0.00000001	" 0.001 "
S 3	S 3 ( " 1).	S 95 ( " S 3).	0.00001	" 0.01 "
S 3	S 3 ( " 1).	S 80 ( " S 3).	0.000001	" 0.001 "
S 3	S 3 ( " 1).	S 149 ( " S 3).	0.000001	" 0.01 "
S 3	S 3 ( " 1).	S 146 ( " S 3).	0.000001	" 0.01 "
S 3	S 3 ( " 1).	S 144 ( " S 3).	0.00001	" 0.001 "
S 3	S 111 (Rabbit 2).	S 3 ( " S 3).	0.0000001	" 0.001 "
S 3	S 118 ( " 3).	S 3 ( " S 3).	0.0000001	" 0.0001 "
S 3	S 2 ( " 4).	S 3 ( " S 3).	0.0000001	" 0.001 "
S 3	S 11 ( " 5).	S 3 ( " S 3).	0.0000001	" 0.001 "
S 3	S 29 ( " 6).	S 3 ( " S 3).	0.00000001	" 0.0001 "
S 3	S 16 ( " 7).	S 3 ( " S 3).	0.00000001	" 0.0001 "
S 3	S 3 ( " 1).	S 107 ( " S 23).	0.0000001	D. 0.0000001 "
S 3	S 3 ( " 1).	S 23 ( " S 23).	0.0000001	" 0.0000001 "
S 3	S 3 (Dog 1).	S 27 ( " S 23).	0.000001	" 0.000001 "
S 3	S 3 ( " 1).	S 67 ( " S 23).	0.00000001	" 0.0000001 "
S 3	S 3 ( " 1).	S 39 ( " S 23).	0.000001	" 0.000001 "
S 3	S 3 ( " 1).	S 75 ( " S 23).	0.000001	" 0.00001 "
S 3	S 3 ( " 2).	S 56 ( " S 23).	0.000001	" 0.000001 "
S 3	S 3 ( " 1).	S 128 ( " S 60).	0.000001	" 0.000001 "
S 3	S 3 ( " 1).	S 60 ( " S 60).	0.000001	" 0.000001 "
S 3	S 3 (Rabbit 1).	S 84 ( " S 84).	0.00000001	" 0.00000001 "
S 3	S 3 ( " 1).	S 1 ( " S 84).	0.000001	" 0.000001 "
S 3	S 3 ( " 1).	S 24 (unclassified).	0.00000001	" 0.00000001 "
S 3	S 3 ( " 1).	S 276 ( " ).	0.0000001	S. 0.0000001 "
S 3	S 3 ( " 1).	S 61 ( " ).	0.00001	" 0.00001 "
S 3	S 3 (Dog 1).	S 152 ( " ).	0.000001	D. 0.000001 "
S 3	S 3 ( " 1).	S 266 ( " ).	0.000001	" 0.000001 "
Unclassified.	S 24 (Rabbit 8).	S 3 (Type S 3).	0.000001	" 0.000001 "
"	S 24 (Sheep 2).	S 14 ( " S 3).	0.00000001	S. 0.00001 "
"	S 276 (Rabbit 9).	S 14 ( " S 3).	0.000001	" 0.000001 "

TABLE XII.

*Summary of the Protective Power of Antistreptococcus Serum, Type S 23, against Strains of the Homologous and Heterologous Types.*

Type of serum.	Strain of <i>S. hemolyticus</i> used for production of serum.	Strain of <i>S. hemolyticus</i> and type used for infection of mice.	Minimal lethal dose of <i>S. hemolyticus</i> used for infection.	Protective power of 0.5 cc. of serum.
			cc.	
S 23	S 23 (Sheep 1).	S 23 (Type S 23).	0.0000001	S. 0.001 cc.
S 23	S 23 ( " 1).	S 107 ( " S 23).	0.0000001	" 0.001 "
S 23	S 23 ( " 1).	S 27 ( " S 23).	0.0000001	" 0.0001 "
S 23	S 23 ( " 1).	S 75 ( " S 23).	0.0000001	" 0.001 "
S 23	S 23 ( " 1).	S 65 ( " S 23).	0.0000001	" 0.001 "
S 23	S 23 ( " 1).	S 3 ( " S 3).	0.0000001	D. 0.000001 "
S 23	S 23 ( " 1).	S 80 ( " S 3).	0.0000001	S. 0.0000001 "
S 23	S 23 ( " 1).	S 55 ( " S 60).	0.000001	D. 0.000001 "
S 23	S 23 ( " 1).	S 60 ( " S 60).	0.000001	S. 0.000001 "
S 23	S 23 ( " 1).	S 128 ( " S 60).	0.000001	D. 0.000001 "
S 23	S 23 ( " 1).	S 50 ( " S 84).	0.0000001	" 0.0000001 "
S 23	S 23 ( " 1).	S 84 ( " S 84).	0.000001	" 0.0000001 "
S 23	S 23 ( " 1).	S 24 (unclassified).	0.000001	" 0.00001 "
S 23	S 23 ( " 1).	S 276 ( " ).	0.000001	" 0.000001 "
Unclassified.	S 24 ( " 2).	S 27 (Type S 23).	0.000001	" 0.000001 "
"	S 24 ( " 2).	S 56 ( " S 23).	0.000001	" 0.000001 "
"	S 24 ( " 2).	S 107 ( " S 23).	0.000001	" 0.000001 "
"	S 24 ( " 2).	S 39 ( " S 23).	0.00000001	" 0.000001 "
"	S 24 ( " 2).	S 23 ( " S 23).	0.00000001	" 0.0000001 "
"	S 276 (Rabbit 9).	S 27 ( " S 23).	0.000001	" 0.000001 "
"	S 276 ( " 9).	S 56 ( " S 23).	0.00001	" 0.000001 "
"	S 276 ( " 9).	S 107 ( " S 23).	0.0000001	" 0.00000001 "
"	S 276 ( " 9).	S 39 ( " S 23).	0.000001	" 0.000001 "
"	S 276 ( " 9).	S 23 ( " S 23).	0.00000001	" 0.00000001 "
"	S 276 ( " 9).	S 67 ( " S 23).	0.000001	" 0.000001 "

TABLE XIII.

*Summary of the Protective Power of Antistreptococcus Serum, Type S 60, against Strains of the Homologous and Heterologous Types.*

Type of serum.	Strain of <i>S. hemolyticus</i> used for production of serum.	Strain of <i>S. hemolyticus</i> and type used for infection of mice.	Minimal lethal dose of <i>S. hemolyticus</i> used for infection.	Protective power of 0.5 cc. of serum.
			cc.	
S 60	S 128 (Rabbit 10).	S 128 (Type S 60).	0.00001	S. 0.01 cc.
S 60	S 128 ( " 10).	S 60 ( " S 60).	0.000001	" 0.0001 "
S 60	S 128 ( " 10).	S 55 ( " S 60).	0.000001	" 0.001 "
S 60	S 128 ( " 10).	S 4 ( " S 60).	0.000001	" 0.001 "
S 60	S 128 ( " 10).	S 72 ( " S 60).	0.00001	" 0.001 "
S 60	S 128 ( " 10).	S 267 ( " S 60).	0.000001	" 0.001 "
S 60	S 128 ( " 10).	S 3 ( " S 3).	0.0000001	D. 0.0000001 "
S 60	S 128 ( " 10).	S 80 ( " S 3).	0.0000001	" 0.0000001 "
S 60	S 128 ( " 10).	S 75 ( " S 23).	0.000001	" 0.00001 "
S 60	S 128 ( " 10).	S 65 ( " S 23).	0.000001	S. 0.00001 "
S 60	S 128 ( " 10).	S 23 ( " S 23).	0.0000001	D. 0.0000001 "
S 60	S 128 ( " 10).	S 84 ( " S 84).	0.000001	" 0.000001 "
S 60	S 128 ( " 10).	S 50 ( " S 84).	0.0000001	" 0.0000001 "
S 60	S 128 ( " 10).	S 24 (unclassified).	0.0000001	" 0.000001 "
S 60	S 128 ( " 10).	S 276 ( " ).	0.000001	" 0.000001 "
Unclassified.	S 24 ( " 8).	S 128 (Type S 60).	0.000001	S. 0.001 "
"	S 276 ( " 9).	S 128 ( " S 60).	0.000001	" 0.0001 "

TABLE XIV.

*Summary of the Protective Power of Antistreptococcus Serum, Type S 84, against Strains of the Homologous and Heterologous Types.*

Type of serum.	Strain of <i>S. hemolyticus</i> used for production of serum.	Strain of <i>S. hemolyticus</i> and type used for infection of mice.	Minimal lethal dose of <i>S. hemolyticus</i> used for infection.	Protective power of 0.5 cc. of serum.	
			cc.		
S 84	S 84 (Sheep 3).	S 84 (Type S 84).	0.000001	S. 0.001	cc.
S 84	S 84 ( " 3).	S 1 ( " S 84).	0.000001	" 0.0005	"
S 84	S 84 ( " 3).	S 20 ( " S 84).	0.000001	" 0.001	"
S 84	S 84 ( " 3).	S 50 ( " S 84).	0.000001	" 0.0005	"
S 84	S 84 ( " 3).	S 139 ( " S 84).	0.000001	" 0.001	"
S 84	S 84 ( " 3).	S 110 ( " S 84).	0.0000001	" 0.001	"
S 84	S 84 ( " 3).	S 15 ( " S 84).	0.0000001	" 0.001	"
S 84	S 1 (Rabbit 11).	S 1 ( " S 84).	0.00000001	" 0.00001	"
S 84	S 1 ( " 11).	S 84 ( " S 84).	0.00000001	" 0.0001	"
S 84	S 1 ( " 11).	S 20 ( " S 84).	0.000001	" 0.001	"
S 84	S 84 (Sheep 3).	S 3 ( " S 3).	0.000001	D. 0.000001	"
S 84	S 84 ( " 3).	S 14 ( " S 3).	0.00000001	S. 0.000001	"
S 84	S 84 ( " 3).	S 23 ( " S 23).	0.00000001	D. 0.0000001	"
S 84	S 84 ( " 3).	S 107 ( " S 23).	0.00000001	S. 0.0000001	"
S 84	S 84 ( " 3).	S 39 ( " S 23).	0.000001	" 0.000001	"
S 84	S 84 ( " 3).	S 67 ( " S 23).	0.000001	D. 0.000001	"
S 84	S 84 ( " 3).	S 27 ( " S 23).	0.000001	" 0.000001	"
S 84	S 84 ( " 3).	S 56 ( " S 23).	0.00001	" 0.000001	"
S 84	S 1 (Rabbit 11).	S 107 ( " S 23).	0.00000001	S. 0.00000001	"
S 84	S 1 ( " 11).	S 128 ( " S 60).	0.000001	D. 0.000001	"
S 84	S 84 (Sheep 3).	S 128 ( " S 60).	0.000001	" 0.000001	"
S 84	S 84 ( " 3).	S 60 ( " S 60).	0.000001	" 0.000001	"
S 84	S 84 ( " 3).	S 276 (unclassified).	0.000001	" 0.000001	"
S 84	S 84 ( " 3).	S 277 ( " ).	0.000001	S. 0.000001	"
S 84	S 84 ( " 3).	S 152 ( " ).	0.000001	D. 0.000001	"
S 84	S 84 ( " 3).	S 266 ( " ).	0.000001	" 0.000001	"
S 84	S 84 ( " 3).	S 24 ( " ).	0.000001	" 0.000001	"
Unclassified.	S 24 (Rabbit 8).	S 84 (Type S 84).	0.00001	" 0.000001	"
"	S 24 (Sheep 2).	S 1 ( " S 84).	0.000001	" 0.000001	"
"	S 276 (Rabbit 9).	S 84 ( " S 84).	0.00000001	" 0.00000001	"
"	S 276 ( " 9).	S 20 ( " S 84).	0.000001	" 0.000001	"
"	S 276 ( " 9).	S 50 ( " S 84).	0.000001	" 0.000001	"



## DISCUSSION.

The complete biological classification of any pathogenic micro-organism presents a very complex problem. The first phase of the undertaking concerns itself with the development of reliable methods for the determination of antigenic differences between members of the species and the application of these methods to the discovery of the immunological relationships between a limited number of strains purposefully selected. In this way the degree of similarity and diversity of type is shown and also the probable number of types, and the proportion of classifiable to unclassifiable strains. The next step of necessity is the testing of the adequacy and universality of the information so gained by applying the tentative classification to a large number of strains of the organism obtained under what may be described as normal conditions of pathogenicity. That some sort of equilibrium has been established in nature among microorganisms that have produced disease over long periods of time is not unlikely. Indeed, evidence obtained from the study of pneumococci supports this view (7), although departure from the norm may occur under special conditions (8). After the relationships of the pathogens of the species to one another have been discovered, it then becomes important for purposes of epidemiological study to compare by the same methods the pathogenic with the saprophytic varieties. This task requires years for its completion and many difficulties and seemingly unexplainable phenomena are encountered. In the beginning, the broader lines of differentiation must be drawn, and divergent results discarded for the time being, since, if the original conception is correct, most of the discrepancies disappear with the advance of knowledge.

In this paper are presented the facts so far obtained in the present study of *Streptococcus hemolyticus* in accordance with the plan outlined above. The strains were collected in a limited community during the course of what may be considered an epidemic of bronchopneumonia secondary to measles. Individuals, however, from all parts of the United States were passing rapidly through this community which was a center for primary training of the aviation service, so that a wider range of territory is represented than the im-

mediate community itself. All the strains were investigated as to their cultural reactions, bile solubility, capacity to hemolyze red blood cells and to ferment the different test sugars, and as to hydrogen ion concentration limiting their growth, and thus identified as accurately as possible as *Streptococcus hæmolyticus* of the human type.

A technique was then developed for studying the immunological reactions of agglutination and protection. By the reaction of agglutination four distinct immunological types and a certain number of unclassifiable strains have been discovered among the 125 strains studied. Individuals of the same type are closely related to one another immunologically, and the different types can be sharply distinguished one from the other. In addition to the four types, study of the reactions of which has been completed, there are in addition two other types, investigation of which is as yet incomplete. The technique of the agglutination reaction demands great care, both as regards the handling of the organisms and the preparation of the medium for their growth. In the medium used by us, a large percentage of strains has grown sufficiently diffusely to permit the preparation of stable suspensions. To what extent continuous growth in this medium has promoted the tendency to diffuseness, and whether the same percentage of freshly isolated strains will grow diffusely, we are as yet unable to say. We have found that by the immunization of sheep a highly specific agglutinating serum is obtained, but that the serum produced from rabbits is not so specific and may show a wider range of crossing, especially in one of the types of streptococcus described. Variations in the specificity of different animal sera have been observed by students of the immunological reactions of meningococcus. In order fully to understand this phenomenon, it would be necessary to compare the specificity of immune sera produced from different species of animals by means of the method of absorption. It is not as yet possible to undertake this kind of an investigation of *Streptococcus hæmolyticus*. The observation has been made, however, that rabbit sera showing non-specific cross-agglutination reactions in general fail to manifest corresponding cross-protection reactions.

Whenever it has been possible to raise the animal virulence of strains of *Streptococcus hæmolyticus*, the evidence obtained from the agglutination tests has been confirmed by that gained from the pro-

tection reaction. In all instances in which this has been done, one reaction has corroborated the findings of the other. The performance of reliable protection tests has been made possible by the production of sufficiently high titer antistreptococcus sera, and by the possibility of raising the animal virulence of a certain number of strains to a high degree. The types of *Streptococcus hæmolyticus* have been noted as Types S 3, S 23, S 60, and S 84, from the serial numbers of the representative strains. This nomenclature is not put forth as a final one, since we realize that probably many other human types exist, to say nothing of the bovine and cheese varieties, and that the proportional distribution of the different varieties pathogenic for man may be very different from that represented by this work. Streptococcus is the largest of all pathogenic groups of bacteria and many years will be required to bring out the information necessary to the perfecting of an adequate classification.

It is of considerable interest that all the members of Type S 60 ferment mannite, and that none of the members of the other groups so far encountered ferments this sugar. A few unclassifiable strains, however, have been found to be mannite fermenters.

This work has cleared up a number of points about *Streptococcus hæmolyticus* which have been in dispute for many years. In the first place, *Streptococcus hæmolyticus* of human origin is not a unit type as was previously supposed, but probably consists of a number of types, at least four of which have been definitely identified. Previous investigators have stated that freshly isolated human strains change their antigenic properties on animal passage, and that the latter procedure for the development of animal virulence gives a common antigenic character to all strains. We have found no evidence to support this contention; in fact immune sera produced with human strains that have never been passed through animals afford a high degree of protection against strains that have received many animal passages. In addition, the antigenic differences between strains of *Streptococcus hæmolyticus* which have been passed through animals are as distinct as those between strains which have not been so passed. The types of *Streptococcus hæmolyticus* studied have been obtained almost exclusively from the respiratory tract and from a limited source of supply, and there is some reason to believe that those which pro-



duce cellulitis, erysipelas, and septicemia may be of somewhat different character. It is, therefore, readily seen that only a beginning has been made in the classification of *Streptococcus hæmolyticus*, and that before the classification is complete and the relative dominance of the different pathogenic varieties determined, much work must be done.

#### SUMMARY.

1. Immunological differences have been shown to exist between strains of *Streptococcus hæmolyticus* of the human type.

2. Four biological types have been identified by means of the reactions of agglutination and protection.

3. At least two other types have been encountered and the indications are that more exist.

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## THE OXYGEN OF THE ARTERIAL AND VENOUS BLOOD IN PNEUMONIA AND ITS RELATION TO CYANOSIS.

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PLATES 13 TO 15.

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In the recent epidemic of influenza with its accompanying pneumonia the unusual frequency of cyanosis was striking, and was in fact one outstanding feature of the epidemic. To find the cause, if possible, of the cyanosis a group of pneumonia cases at the Hospital of The Rockefeller Institute was studied during the past winter, with particular reference to cyanosis and its relation to the arterial and venous blood oxygen.

Studies of the venous blood have been made, notably by Lundsgaard<sup>1</sup> in cardiac insufficiency, and Harrop<sup>2</sup> in pneumonia; but the interpretation of the results is difficult, because of the undeterminable factors which affect the venous oxygen, such as variations in the rates of circulation and metabolism in the parts from which the blood is drawn. It appeared that satisfactorily complete data on which to base an explanation of the cyanosis could be expected only from analyses of arterial as well as venous blood. A group of 33 cases of pneumonia is here presented in which the oxygen of both arterial and venous blood has been determined.

### *Method.*

*Technique of Arterial Puncture.*—Hürter<sup>3</sup> has shown that puncture of the radial artery is a safe procedure. At the Hospital of

<sup>1</sup> Lundsgaard, C., *J. Biol. Chem.*, 1918, xxxiii, 133; *J. Exp. Med.*, 1918, xxvii, 179, 219.

<sup>2</sup> Harrop, G. A., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 10.

<sup>3</sup> Hürter, *Deutsch. Arch. klin. Med.*, 1912, cviii, 1.

The Rockefeller Institute arterial puncture has been done six times on one patient without injury. In all, about 90 punctures have been done and no ill results have been observed. The possible dangers are hemorrhage, thrombosis, embolism, or aneurysm, but in this series these have never been observed, even after 4 to 5 months observation. Occasionally if proper precautions are not observed, an undue amount of blood extravasation occurs; this has been observed here once, but the blood was rapidly absorbed and no after effects were seen.

An ordinary 20 cc. all glass Luer syringe is used with a Luer needle 1 to 2 mm. in diameter. The point of the needle is beveled at an angle of about  $45^{\circ}$  and must be very sharp. To prevent the blood from coming in contact with air, 1 or 2 cc. of sterile albolene are poured into the barrel of the syringe, the plunger is inserted, and the syringe with the attached needle inverted. The plunger is forced upward, and the air in the dead space at the distal end of the syringe and needle is expelled. The excess of albolene is then forced out so that only a small amount remains in the needle and in the small dead space. The patient's arm is laid horizontally upon a pillow, the hand is flexed backwards, and the region over the radial artery is sterilized with tincture of iodine. The end of the left index finger of the operator is then sterilized with iodine and, by using this finger in palpation, the best site for the puncture is determined. (Since the position of the artery is determined solely by palpation, it is advantageous to avoid gloves and use the bare finger.) The skin at the site of the proposed puncture is anesthetized with novocaine. The syringe and needle are held at an angle of about  $45^{\circ}$  to the surface, the needle is then pushed through the skin, and, after carefully relocating its position, the needle is entered into the artery. It is essential that the position of the artery should be sharply located and the point of maximum pulsation chosen (usually opposite the radial styloid); then the artery is easily entered, the pressure of the blood stream forces up the plunger so that suction is unnecessary, and within 15 to 60 seconds from 10 to 20 cc. of blood can be obtained. The needle is then quickly withdrawn, and by means of a compress, firm pressure is immediately applied over the artery for 1 or 2 minutes, so as to obliterate it temporarily and prevent extravasation. The

wrist is then bandaged with three or four thicknesses of compress to get greater pressure, and at the end of about 2 to 3 hours the bandage may be removed. If the artery is missed at the first puncture, and especially if a hematoma begins to form or blood extravasates around the needle, the operator should desist at once.

The blood thus collected is transferred to a tube 2.5 by 10 cm. in which a layer of albolene at least 2 cm. deep has previously been placed (to prevent contact with air) with some potassium oxalate to prevent coagulation.

Not all cases are suitable for arterial punctures, especially where repeated punctures are contemplated. Many women and some men have small radial arteries deeply situated, and hence difficult to puncture. The more rapid and bounding the pulse, the easier the puncture; and cases with pulse rates of 70 or below are difficult. Occasionally when the artery has been touched with the point of the needle it becomes almost pulseless, due no doubt to reflex vasoconstriction, but after 15 to 60 seconds it relaxes and the puncture may be finished.

Following the puncture there is a numbness of the radial side of the hand, but this wears off rapidly. About 50 per cent of the subjects complain of very slight dull pain at the wrist lasting about 12 to 24 hours, but this is without significance.

In two cases which were autopsied the radial arteries, which had been punctured several times, were dissected out. In one case the sites of the punctures were difficult to determine, and there was only a small amount of extravasation of blood. In the second case one puncture had been made 4 or 5 hours before death, when the patient was almost pulseless and the puncture difficult; here there was a moderate amount of extravasated blood in the tissues surrounding the artery, and the site of the puncture was marked by a pin-head point of extravasation of blood in the wall of the artery.

*Technique of Venous Puncture.*—The venous blood was obtained without stasis by the technique devised by Lundsgaard.<sup>1</sup>

*Determination of Oxygen Content, Oxygen Capacity, and Oxygen Unsaturation.*<sup>1</sup>—The method of Van Slyke<sup>4</sup> was used. The arterial

<sup>4</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1918, **xxxiii**, 127.



TABLE I.  
*Determination of Oxygen Content, Oxygen Capacity, and Oxygen Unsaturation.*

Case No.	Day of disease.	Diagnosis and clinical notes.	Result.	Cyanosis.					Oxygen content.		Oxygen capacity per 100 cc. of blood.	Oxygen unsaturation.			
				General.	Cheeks.	Nose.	Lips.	Ears.	Fingers.	Arterial, per 100 cc. of blood.	Venous, per 100 cc. of blood.	Arterial.	Per 100 cc. of blood.	Per cent.	Venous.
3	6th	Lobar pneumonia.	R.*	0†	0	0	0	0	0	cc.	cc.	cc.	1.7	8.5	cc.
	8th	Acutely ill.		0	0	0	0	0	0	18.4	14.8	20.1†	2.4	11.9	5.3
	11th			2	1	0	0	0	2	17.7	15.0	20.1†	2.9	14.1	5.1
	14th	After crisis; much better.		1	1	0	0	0	1	19.1	17.5	21.0	1.9	9.0	3.5
	20th	Convalescing.		0	0	0	0	0	0	18.9	15.1	20.1	1.2	6.0	5.0
4	30th	"		0	0	0	0	0	0	19.9	17.8	19.9	0.0	0.0	2.1
	7th	Influenza; bronchopneumonia. Extremely ill.	D.	3	3	2	1	1	3	15.1	5.3	20.3	5.2	25.6	15.0
5	10th	Influenza; bronchopneumonia. Heart's blood, 5 p.m.	"	4	4	4	4	4	4						19.6
				During illness.											84.5
8	24th	Influenza; bronchopneumonia.	R.	0	0	0	0	0	0	17.1	12.0	18.6	1.5	8.1	6.6
	9th	Bronchopneumonia.	D.	4	4	3	1	2	4	7.9	3.6	24.8	16.9	68.2	21.2
10	18th	"	R.	0	0	0	0	0	0	22.7	18.5	23.8	1.1	4.6	5.3
															22.3
11	7th	Influenza; bronchopneumonia; pleuritic effusion.	"	2	2	0	0	1	2	23.1	20.1	25.7	2.6	10.1	5.6
	22nd			0	0	0	0	0	0	21.5	16.1	23.6	2.1	8.9	7.5

14	9th	Lobar pneumonia; influenza.	D.	3	2	1	1	1	1	3	21.1	10.6	26.6	5.5	20.7	16.0	60.2
15	3rd 11th	Influenza; bronchopneumonia.	R.	1	1	0	0	0	0	1	20.0	16.7	21.7	1.7	7.8	5.0	23.0
				0	0	0	0	0	0	0	19.1	16.4	20.6	1.5	7.3	4.2	20.4
16	4th	Bronchopneumonia.	D.	3	3	2	0	2	3	20.7	13.8	24.1	3.4	14.1	10.3	42.8	
17	13th 15th	Lobar pneumonia.	R.	1	1	0	0	0	1	16.9	16.2	20.2	3.3	16.3	4.0	19.8	
				1	1	1	1	0	0	16.1	9.0	18.2	2.1	11.5	10.2	56.0	
18	23rd 24th 26th	" Influenza; empyema.	D.	2	2	1	1	0	2	19.6	17.7	23.5	3.9	16.6	5.8	24.7	
				3	3	2	1	2	3	19.8	17.1	23.7	3.9	16.5	6.6	27.8	
				4	4	3	2	3	4	14.4	1.3§	23.3	8.9	38.2	22.0	94.5§	
19	4th 5th 13th	" " bronchopneumonia.	R.	0	0	0	0	0	0	21.0	17.5	22.8	1.8	7.9	5.3	23.2	
				0	0	0	0	0	0	21.7	17.2	23.4	1.7	7.3	6.2	26.5	
				0	0	0	0	0	0	20.7	17.2	20.7	0.0	0.0	3.5	16.9	
20	11th	"	"	1	1	0	1	0	1	18.1	15.7	20.9	2.8	13.4	5.2	24.9	
21	7th 7th	" Heart puncture (p.m.; 1½ hrs.).	D.	4	4	4	2	3	4	10.8	4.7	22.5	11.7	52.0	17.8	79.2	
											1.8	22.5			20.7		
22	11th 13th	Lobar pneumonia.	"	2	2	1	1	0	2	18.6	9.3	23.1	4.5	19.5	13.8	59.8	
				2	2	1	1	1	2	16.7	12.7	22.3	5.6	25.1	9.6	43.0	

\* R. indicates recovered, D. died.

† The numbers in this column indicate plus signs; i. e., 0 indicates no cyanosis, 1 indicates + (slight cyanosis), 2 indicates ++ (moderate cyanosis), 3 indicates +++ (marked cyanosis), and 4 indicates ++++ (intense cyanosis).

‡ Determined colorimetrically (Palmer method—Palmer, W. W., *J. Biol. Chem.*, 1918, xxxiii, 119).

§ Postmortem heart's blood.

TABLE I—*Concluded.*

Case No.	Day of disease.	Diagnosis and clinical notes.	Result.	Cyanosis.						Oxygen content.		Oxygen capacity per 100 cc. of blood.	Oxygen unsaturation.			
				General.	Cheeks.	Nose.	Lips.	Ears.	Fingers.	Arterial, per 100 cc. of blood.	Venous, per 100 cc. of blood.		Arterial.		Venous.	
													Per 100 cc. of blood.	Per cent.	Per 100 cc. of blood.	Per cent.
23	6th 7th	Influenza; bronchopneumonia.	R.	1	1	1	1	0	1	cc. 18.5	cc. 11.3	cc. 21.8	cc. 3.3	15.1	cc. 10.5	48.2
24	6th 11th 25th	"	"	1	1	1	1	0	1	21.6	9.2	23.7	2.1	8.9	14.5	61.2
25	9th	"	D.	3	3	2	1	2	3	15.7	12.6	28.1	12.4	44.1	15.5	55.2
29	14th	Lobar pneumonia.	R.	0	0	0	0	0	0	19.9	14.8	20.4	0.5	2.5	5.6	27.5
30	6th	Influenza; bronchopneumonia.	D.	3	3	1	2	2	3	17.2	7.9	22.9	5.7	24.9	12.0	48.3
31	4th 9th	Multiple pulmonary abscesses; empy- ema.	"	2	1	1	0	1	2	17.1	15.7	20.2	3.1	15.3	4.5	22.3
32	6th 7th	Influenza; bronchopneumonia.	"	1	0	0	0	0	2	17.8	19.6	24.7	1.8	9.2	15.0	60.8
33	8th	"	"	3	2	2	1	2	4	19.9	11.8	26.2	6.3	24.0	14.4	55.0
34	10th 13th	Bronchopneumonia.	"	2	1	1	0	1	2	17.0	13.5	22.2	5.2	23.4	8.7	39.2
			"	3	3	1	1	2	3	12.9	17.1	16.2	4.2	24.6		
				3	3	2	1	2	3	11.3			4.8	29.6		





and venous samples were taken under albolene to prevent contact with air and analyzed in duplicate immediately for oxygen content. A portion of the blood was saturated with oxygen and the total oxygen capacity determined, as described by Van Slyke. Thus are obtained (1) arterial oxygen content (cubic centimeters of oxygen combined with hemoglobin in 100 cc. of arterial blood), (2) venous oxygen content (a similar value for venous blood), (3) total oxygen capacity (cubic centimeters of oxygen combined with the hemoglobin of 100 cc. of blood when fully saturated).

The difference between oxygen content and total oxygen capacity has been named by Lundsgaard<sup>1</sup> the oxygen unsaturation, and we have followed his usage of the term. The unsaturation may be expressed either as cubic centimeters of oxygen per 100 cc. of blood, or as percentage of the total oxygen capacity. In the latter case the data represent the per cent of total hemoglobin in the form of reduced hemoglobin. An example will make this clear.

	cc.	per cent
Arterial oxygen content.....	18.0	90.0
Venous oxygen content.....	14.0	70.0
Total oxygen capacity.....	20.0	100.0
Arterial oxygen unsaturation.....	2.0	10.0
Venous oxygen unsaturation.....	6.0	30.0

#### RESULTS.

Table I gives the results obtained in the 33 cases of pneumonia studied.

38 cases were studied which were divided as indicated in Table II.

TABLE II.  
*Classification of Cases.*

Diagnosis.	No
Pneumonia, lobar.....	7
Postinfluenzal bronchopneumonia .....	25
Multiple pulmonary abscesses.....	1
Normal individuals .....	5
Total.....	38
Complications, empyema.....	3

*Results in Normal Controls.*—Table III gives the results obtained in five normal resting men. All these subjects were up and about, but were punctured 15 to 30 minutes after resting in bed.

The range of arterial oxygen content is from 17.9 to 22.1 cc. per 100 cc. of blood. The arterial unsaturation varies from 2.8 to 6.3 per cent. The venous unsaturation varies from 22.7 to 33 per cent. The arterial blood is usually assumed to be approximately saturated, but in the five individuals given in Table III the mean value is 95 per cent. The values found for the venous oxygen unsaturation are in close accord with those of Lundsgaard in normal individuals.

TABLE III.

*Arterial and Venous Oxygen, Total Oxygen Capacity, and Arterial and Venous Oxygen Unsaturation in Five Normal Individuals.*

Individual No.	Oxygen content.		Oxygen capacity per 100 cc. of blood.	Unsaturation.			
	Arterial, per 100 cc. of blood.	Venous, per 100 cc. of blood.		Arterial.		Venous.	
				Per 100 cc. of blood.	Per cent.	Per 100 cc. of blood.	Per cent.
	cc.	cc.	cc.	cc.		cc.	
1	17.9	12.8	19.1	1.2	6.3	6.3	33.0
2	21.0	16.7	21.6	0.6	2.8	4.9	22.7
3	22.1	17.2	23.3	1.2	5.2	6.1	26.2
4	20.2	15.6	21.6	1.4	6.5	6.0	27.8
5	19.5	15.4	20.3	0.8	3.9	4.9	24.1
Mean.....	20.2	15.6	21.2	1.0	5.0	5.6	26.8

*Arterial and Venous Oxygen in the Pneumonia Cases.*—That the arterial and venous oxygen content and unsaturation in the pneumonia cases show striking contrasts to the normal individuals is seen at once from Table IV.

TABLE IV.

*Maximum and Minimum Arterial and Venous Oxygen Content and Arterial and Venous Oxygen Unsaturation in Pneumonia Cases.*

Arterial content per 100 cc. of blood.		Arterial unsaturation.		Venous content per 100 cc. of blood.		Venous unsaturation.	
Maximum.	Minimum.	Maximum.	Minimum.	Maximum.	Minimum.	Maximum.	Minimum.
cc.	cc.	per cent	per cent	cc.	cc.	per cent	per cent
22.9	7.9	68.2	0.0	20.1	3.6	85.5	14.4

The arterial oxygen unsaturation ranges up to 68.2 per cent as contrasted to 6.3 per cent for normal individuals. For the venous unsaturation the variations are just as marked.

*Oxygen Unsaturation in Fatal and Non-Fatal Cases.*—If the cases are divided according to the outcome, equally characteristic differences are obtained. Table V shows that the maximum arterial unsaturation

TABLE V.

*Arterial and Venous Oxygen Unsaturation in Sixteen Non-Fatal Cases.*

Case No.	Maximum* unsaturation.	
	Arterial.	Venous.
	<i>per cent</i>	<i>per cent</i>
3	14.1	60.1
8	8.1	35.5
10	4.6	22.3
11	10.1	31.8
15	7.8	23.0
17	16.3	56.0
19	9.8	26.5
20	13.4	24.9
23	15.1	61.2
24	4.0	45.7
29	2.5	27.5
35	33.0	46.7
36	13.3	21.3
37	7.5	14.4
38	8.4	34.2
39	14.5	39.7
Mean .....	13.9	36.3

\* Where more than one determination was made the maximum observed value is given.

in the recovered cases is 33 per cent, while the mean value (13.9 per cent) is more than twice the normal mean. In one case (No. 35), however, the arterial unsaturation was 33 per cent. This patient was desperately ill and the outcome for some time appeared hopeless, but she made a remarkable recovery.

Table VI shows that the arterial unsaturation in the fatal cases is much greater, the mean value being 32 per cent. Fourteen of the

sixteen fatal cases, but only one of sixteen non-fatal cases, had an arterial unsaturation greater than 20 per cent. Hence the fatal outcome of pneumonia is usually associated with a great degree of arterial unsaturation, and the arterial oxygen unsaturation offers a valuable prognostic sign. Rarely does a patient with a value greater than 20 per cent recover (one case out of 33).

A study of the venous unsaturation shows a far less degree of uniformity, and indicates the presence of factors difficult to control,

TABLE VI.

*Arterial and Venous Oxygen Unsaturation in Sixteen Fatal Cases.*

Case No.	Maximum* unsaturation.	
	Arterial.	Venous.
	<i>per cent</i>	<i>per cent</i>
4	25.6	73.9
9	68.2	85.5
14	20.7	60.2
16	14.1	42.8
18	38.2	
21	52.0	79.2
22	25.1	59.8
25	44.1	55.2
30	24.9	48.3
31	15.3	22.3
32	24.0	60.8
33	23.4	39.2
34	29.6	
40	27.3	
41	27.6	
42	54.5	
Mean.....	32.0	57.0

\* Where more than one determination was made the maximum observed value is given.

which make the determination of the venous unsaturation of less prognostic significance than the arterial. However, in the fatal cases the mean value is 20 per cent higher than in the non-fatal cases. Eight of the eleven cases, or 72 per cent, showing a venous unsaturation over 47 per cent were fatal, while thirteen out of sixteen, or 81 per cent, showing less than 47 per cent venous unsaturation recovered.



Table VII gives briefly a summary of the above discussion with the maximum and minimum arterial and venous oxygen unsaturation in the fatal and non-fatal cases.

TABLE VII.

*Maximum and Minimum Arterial and Venous Oxygen Unsaturation in Fatal and Non-Fatal Cases.*

Type of case.	No. of cases.	Arterial unsaturation.			Venous unsaturation.		
		Maximum.	Minimum.	Mean.	Maximum	Minimum.	Mean.
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Fatal cases.....	16	68.2	14.1	32.0	85.5	22.3	57.0
Non-fatal cases.....	16	33.0	1.6	13.9	61.2	14.4	36.3
Normal individuals..	5	6.5	2.8	5.0	33.0	22.7	26.8

### *Cyanosis.*

Cyanosis may be caused by one of three factors or any combination of these three.

1. *Disturbance of the Capillary Bed.*—If there is a constriction of the arterial precapillaries, or a dilatation of the capillaries due to any cause, there is a stagnation of blood in the capillaries which, in the superficial or distal parts of the body, such as the lips, nose, ears, or fingers, gives cyanosis. For example, the cyanosis after prolonged exposure to cold is undoubtedly due to this cause. Cyanosis associated with various vasomotor paralyses, as in hemiplegia and poliomyelitis, may also be explained in this way.

2. *Change in the Hemoglobin.*—Certain intoxications change the hemoglobin into substances which do not contain labile oxygen, and therefore reduce the oxygen capacity of the blood. These substances are methemoglobin and sulfhemoglobin, and they are found in the blood associated with the cyanosis of acetanilide, phenacetin, potassium chlorate, or nitrobenzol poisoning.<sup>5</sup>

Enterogenous cyanosis due to methemoglobin or sulfhemoglobin, and cases of cyanosis in which the presence of these substances can

<sup>5</sup> Hammarsten, O., Text book of physiological chemistry, New York, 7th edition, 1914, 283.

be shown, and in which there is a reduction of the total oxygen capacity of the blood, would fall into this class.

It is possible that the cyanosis associated with pneumonia, more particularly in the very severe cases with a marked septicemia, may in some measure be caused by this factor. Butterfield and Peabody<sup>6</sup> have shown that the growth of pneumococci *in vitro* results in the formation of a substance with the optical properties of methemoglobin. Further, Peabody,<sup>7</sup> in rabbits in which he induced an overwhelming septicemia with pneumococci, found a rapid and marked fall in the total oxygen capacity of the blood, and also observed that the blood was of a brownish color and took up oxygen slowly. However, he was rarely able to demonstrate the presence of methemoglobin in such blood, and it may be pointed out that such overwhelming septicemias as were produced in his rabbits (direct films of the blood showed numerous organisms) are never found in man. Peabody<sup>8</sup> and Harrop<sup>2</sup> found, in a few severe cases of pneumonia, a diminution in the total oxygen capacity of the blood, but were unable to demonstrate the presence of methemoglobin in the blood.

3. *Admixture Cyanosis*.—The third factor in the production of cyanosis is a deficient or incomplete oxygenation of the blood as it passes through the pulmonary capillaries, or the passage of but part of the blood through the lungs (as in congenital heart disease), so that there is an abnormally low percentage of oxyhemoglobin in the peripheral circulation. Obviously the study of the arterial blood oxygen would determine this.

*Degree of Cyanosis and the Oxygen Unsaturation*.—Cyanosis, to a greater or less degree, was observed in 28 cases out of 33. In order to compare cyanosis in different parts from time to time the following scale was used.  $\pm$  indicates very slight, + slight, ++ moderate, +++ marked, and ++++ intense cyanosis.

In the five cases (Nos. 8, 10, 19, 24, and 29) of pneumonia in which no cyanosis was observed at any time, the maximum arterial un-

<sup>6</sup> Butterfield, E. E., and Peabody, F. W., *J. Exp. Med.*, 1913, xvii, 587.

<sup>7</sup> Peabody, F. W., *J. Exp. Med.*, 1913, xviii, 1.

<sup>8</sup> Peabody, F. W., *J. Exp. Med.*, 1913, xviii, 7.

saturation was 8.1 per cent, slightly greater than normal (Table VIII). The mean value is 5.4 per cent, only 0.4 per cent greater than for the normal individuals. The venous unsaturations follow in the same order, but are more irregular.

In the twenty-seven cases showing cyanosis during their illness (Table IX), the difference is at once apparent. Here the variations are from 7.3 to 68.2 per cent of arterial unsaturation, and all but three of the values are above 10 per cent. The mean, 24.7 per cent, is five times greater than in normal individuals. Similarly the values

TABLE VIII.

*Maximum\* Arterial and Venous Oxygen Unsaturation in Cases without Cyanosis.*

Case No.	Arterial unsaturation.		Venous unsaturation.	
	Per 100 cc. of blood.	Per cent.	Per 100 cc. of blood.	Per cent.
	cc.		cc.	
8	1.5	8.1	6.6	35.5
10	1.1	4.6	5.3	22.3
19	1.8	7.9	6.2	26.5
24	0.8	4.0	9.6	45.7
29	0.5	2.5	5.6	27.5
Mean.....	1.1	5.4	6.7	31.5

\*Where more than one determination was made the maximum observed value is given.

for the venous unsaturation are higher and the mean, 44.5 per cent, is greater than in the cases without cyanosis.

Not only is there a greatly increased arterial and venous unsaturation in the cases with cyanosis, but there is a definite relation between the degree of cyanosis and the degree of unsaturation. In Table X the observations are divided into five groups according to whether there was no, slight, moderate, marked, or intense cyanosis at the time the blood was obtained. There is a gradual increase in both arterial and venous unsaturation as the associated cyanosis increases. This is strikingly brought out by Text-fig. 1 which shows as a curve the relation between unsaturation and cyanosis. The curve for the venous unsaturation parallels that for the arterial unsaturation.

TABLE IX.

*Maximum\* Arterial and Venous Oxygen Unsaturation in Cases with Cyanosis.*

Case No.	Arterial unsaturation.		Venous unsaturation.	
	Per 100 cc. of blood.	Per cent.	Per 100 cc. of blood.	Per cent.
	<i>cc.</i>		<i>cc.</i>	
3	2.9	14.1	5.3	26.4
4	5.2	25.6	15.0	73.9
9	16.9	68.2	21.2	85.5
11	2.6	10.1	5.6	21.8
14	5.5	20.7	16.0	60.2
15	1.7	7.8	5.0	23.0
16	3.4	14.1	10.3	42.8
17	3.3	16.3	10.2	56.0
18	8.9	38.2	6.6	27.8
20	2.8	13.4	5.2	24.9
21	11.7	52.0	17.8	79.2
22	5.6	25.1	13.8	59.8
23	3.3	15.1	14.5	61.2
25	12.4	44.1	15.5	55.2
30	5.7	24.9	12.0	48.3
31	3.1	15.3	4.5	22.3
32	6.3	24.0	15.0	60.8
33	5.2	23.4	8.7	39.2
34	4.8	29.6		
35	6.3	33.0	8.9	46.7
36	3.5	13.3	5.6	21.3
37	1.6	7.3	3.2	14.4
38	1.6	8.4	6.5	34.2
39	3.1	14.5	8.5	39.7
40	6.1	27.3		
41	7.9	27.6		
42	9.7	54.5		
Mean.....	5.6	24.7	10.2	44.5

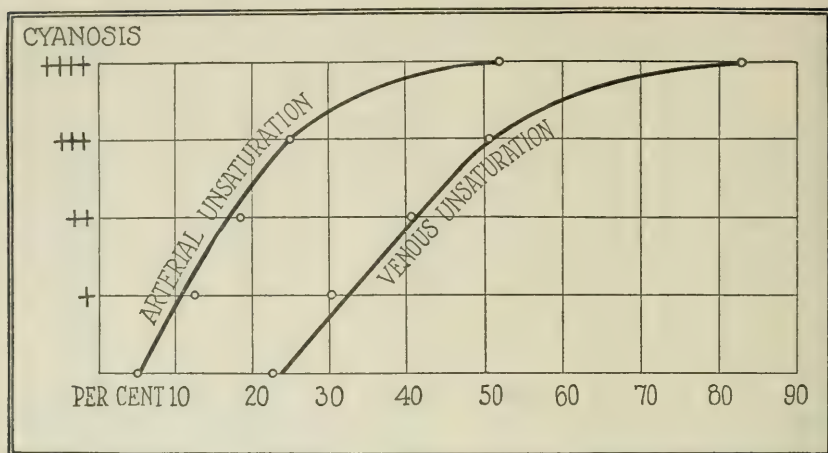
\* Where more than one determination was made the maximum observed value is given.

TABLE X.

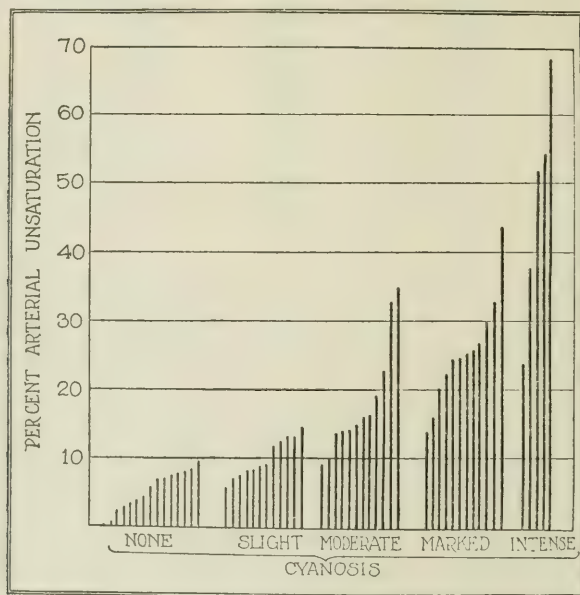
*Arterial and Venous Oxygen Unsaturation Associated with Cyanosis of Varying Degree.*

Cyanosis.	No. of observations.	Unsaturation.	
		Mean arterial.	Mean venous.
		<i>per cent</i>	<i>per cent</i>
None.....	18	5.8	23.8
Slight.....	11	11.8	30.4
Moderate.....	10	17.2	41.8
Marked.....	13	26.0	51.2
Intense.....	4	53.2	82.3





TEXT-FIG. 1. Curves showing the relation between arterial and venous unsaturation and degree of cyanosis.



TEXT-FIG. 2. The observations of the per cent arterial unsaturation are plotted in groups according to the degree of cyanosis at the time of observation. The increase of the per cent oxygen unsaturation of the arterial blood with increasing cyanosis is striking.

In Text-fig. 2 the individual values for each observation are plotted in groups according to the degree of cyanosis. Here again the steadily increasing unsaturation with increasing cyanosis is apparent.

Again the relation of cyanosis to blood unsaturation is shown by a study of cases, in which repeated determinations were made on the same patient at different stages of the disease and with varying degrees of cyanosis.

Case 3 (Text-fig. 3) at the first observation (6th day) had no cyanosis, the arterial unsaturation being 8.5 per cent. He became much sicker and had a moderate cyanosis on the 11th day. The arterial unsaturation was then 14.1 per cent. With recovery the cyanosis disappeared, and on the 30th day the arterial unsaturation was 0.0 per cent.



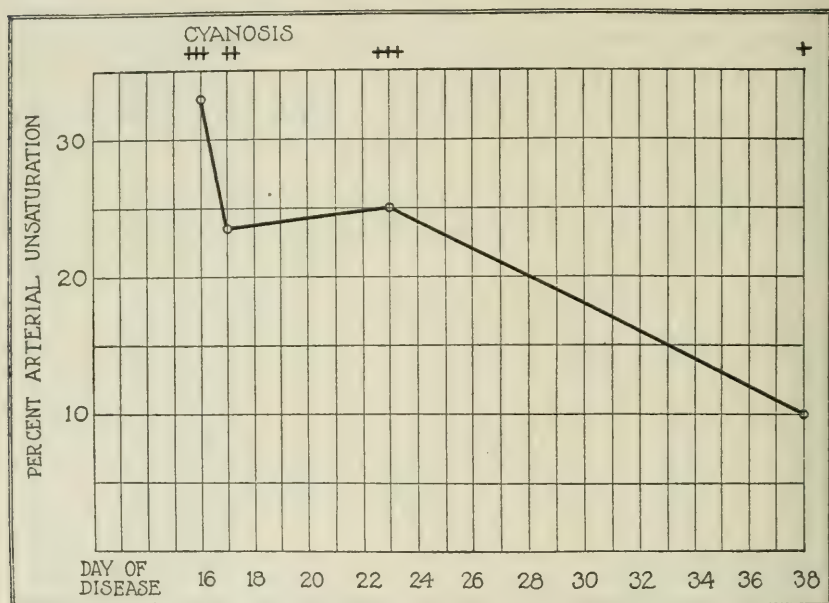
TEXT-FIG. 3. Curve of arterial oxygen unsaturation in Case 3. Note the increase in both cyanosis and arterial unsaturation as the patient became worse. After recovery the cyanosis disappeared and the arterial unsaturation became 0 per cent.

Case 35 (Text-fig. 4) was critically ill and markedly (++++) cyanotic on the 16th day; the arterial unsaturation was 33 per cent. For some time the outlook was desperate and the cyanosis continued associated with an arterial unsaturation of 23.5 and 25.9 per cent on the 17th and 23rd days. Subsequently the patient became much better; the cyanosis diminished until it was slight. The arterial unsaturation was then 10.7 per cent. After complete

recovery (95th day) when no cyanosis was present, the arterial unsaturation was 6.7 per cent.

On the other hand, Case 18 increased the arterial unsaturation from 16.6 per cent to 38.2 per cent as he became worse and the cyanosis increased (Text-fig. 5).

*Discussion of Color.*—The comparison of the colors of the cyanotic parts with the standard colors in “*Répertoire de couleurs*”<sup>9</sup> shows



TEXT-FIG. 4. Curve of arterial oxygen unsaturation in Case 35. There is a gradual decrease of the arterial unsaturation as the patient became better and the cyanosis diminished.

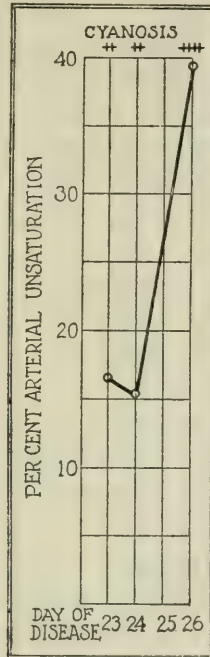
that there is considerable variation in the shades. The basic color is blue, but due to the varying admixtures with red, shades of heliotrope and mauve are frequent, especially in the fingers. On the cheeks a reddish heliotrope is not uncommon, but when the facial cyanosis is diffuse, the color is a leaden or plumbago-blue.

*Distribution of Cyanosis.*—The most constant and frequent site of the cyanosis was in the end of the fingers, especially under the

<sup>9</sup> Oberthür, R., *Répertoire de couleurs*, 1905.

nails. In cases with slight cyanosis a faint but definite bluish tinge could be observed here when no cyanosis could be made out elsewhere.

As the cyanosis became more intense it could next be observed over the entire end of the finger, being more marked on the dorsal aspect and fading gradually toward the first joint and toward the palmar surface.



TEXT-FIG. 5. Curve of arterial oxygen unsaturation in Case 18. The cyanosis increased from ++ to +++ in 3 days, and the arterial unsaturation increased from 16.6 per cent to 38.2 per cent.

Fig. 1 shows the hand in Case 39 with a moderate (++) degree of cyanosis. This corresponded to 14.5 per cent of arterial unsaturation. The cyanosis is confined to the finger-nails and is of the heliotrope shade. In this case there was cyanosis of the lips, nose, and cheeks, but the color was less intense than in the finger-nails.

Fig. 2 shows the hand of a case with intense (++++ ) cyanosis with an arterial unsaturation of 44.1 per cent. The tips of the



fingers are a darker mauve-blue which is most intense under the nails. On the dorsum the color fades gradually beyond the terminal joint and also toward the palmar aspect.

The cyanosis of the toes is of the same order, but as a rule much less marked.

Second to the fingers the face shows cyanosis. Here it is of different types and distribution. At times even with a high degree of unsaturation, as in Case 34 (Fig. 3) with a marked (+ + +) degree of cyanosis, there is only a slight, dull, leaden blue, diffusely spread over the face from the forehead to and including the chin. In this case the fingers were darkly cyanotic (+ + +) and yet the face shows no marked accumulation of color at any one area, and seen alone would not lead one to suspect so high a degree of unsaturation.

Case 31 (Fig. 4) illustrates a second type. Here the 15.3 per cent of arterial unsaturation is accompanied by facial cyanosis in which the color is not diffuse but localized to the areas most frequently involved; namely, nose, chin, lips, ears, and cheeks.

When the cyanosis is slight it may be most marked over the malar bone. Frequently, despite a high arterial unsaturation and deep cyanosis of the fingers, the cyanosis is sharply limited to this area of the face and is of a dull cherry-red-blue at the center surrounded by a fading band of color similar to that seen in the fingers. Fig. 5 is an illustration of this type.

Even with a high arterial unsaturation the chin infrequently shows cyanosis, and the lips and ears, but occasionally. In fact the rarity and lesser degree of cyanosis of the lips in the pneumonia cases is in striking contrast to its frequency in cardiac cases.

Facial cyanosis is characterized also by its variability from day to day and from hour to hour. Often marked changes occurred within an hour. Change of position and coughing produced great changes in the intensity of the facial cyanosis. Therefore, as a measure of the degree of cyanosis from time to time the fingers are the best guide, for here the cyanosis remains most constant.

*Total Oxygen Capacity.*—A consideration of the oxygen capacity is important to determine whether there is methemoglobin production in pneumonia. In the entire series the total capacity varied from 14.9 to 28.6 cc. In the non-fatal cases (Table XI) there is

no unusually low capacity (except in Case 35) and the mean, 20.0 cc., is slightly lower than the normal mean. In Case 35 there was a drop in 6 days from 19.1 cc. to 15.8 cc. This patient's blood culture was negative and she was desperately ill with marked cyanosis. No examination was made for methemoglobin and the cause of this sudden drop is undetermined.

TABLE XI.  
*Oxygen Capacity of Non-Fatal Cases.*

Case No.	Oxygen capacity per 100 cc. of blood.	Degree of cyanosis.
	cc.	
3	19.9	++
8	18.6	0
10	18.5	0
11	23.6	++
15	20.6	+
17	17.5	+
19	17.2	0
20	20.9	+
23	21.8	+
24	18.6	0
29	20.4	0
35	14.9	+++
36	26.3	+
37	21.8	+
38	19.0	+
39	21.4	++
Mean.....	20.0	

In the fatal cases (Table XII) all had marked cyanosis and should show low capacities if the formation of methemoglobin played an important part in the cyanosis. However, values even slightly below normal were shown only by Case 34. This patient was cyanotic throughout, and during a 3 day period her capacity decreased 0.9 cc. Her blood culture was sterile. With the exception of this the capacities are high and the mean, 23 cc., is higher than the normal.

Again a consideration of the changes in total oxygen capacity (Table XIII) shows that for the fatal cases there was during the illness but slight loss, and many cases show a gain. These changes

are no greater than for the non-fatal cases (Table XIV), particularly those which showed no cyanosis. Case 35 was the only case of the series which showed an unusual fall of capacity (4.2 cc.) associated with marked cyanosis.

It may be said, however, that but four cases, two of which were fatal, had positive blood cultures of pneumococci. Only one (No.

TABLE XII.  
*Oxygen Capacity of Fatal Cases.*

Case No.	Time before death.	Oxygen capacity (gasometric) per 100 cc. of blood.	Degree of cyanosis.
		"	
4	7 hrs.	20.3	+++
5	Heart puncture (p.m.).	23.2	++++ during ill- ness.
9	20 min.	24.8	+++
14	2 days.	26.6	+++
16	4 "	24.1	+++
18	3 "	23.7	+++
21	8 hrs.	22.5	+++++
22	12 "	22.3	++
25	1 day.	28.1	+++
30	1 "	22.9	+++
31	6 days.	19.6	++
32	1 day.	26.2	+++
33	1 "	22.2	++
34	1 "	16.2	+++
40		22.3	+++
41		28.6	+++
42		17.8	+++++
Mean .....		23.0	

22) had an infinite number of colonies per cc. of blood; the others had but a few. This might explain why, unlike Peabody and Harrop, we failed to find occasional cases with greatly reduced oxygen capacity.

It seems unlikely, therefore, that methemoglobin formation plays any important part in the production of the cyanosis here observed, or had any part in the fatal outcome of the sixteen cases.

A striking feature is the unusually high values for the oxygen capacities of some of the very ill or fatal cases. Cases 9, 14, 16, 25, 32, and 41 show this characteristic, but the exact cause of this high capacity is still unknown.

TABLE XIII.

*Change in Oxygen Capacity in Fatal Cases.*

Case No.	Change in capacity per 100 cc. of blood.	Interval.
	<i>cc.</i>	<i>days</i>
18	-0.2	3
22*	-0.8	2
31	-0.6	5
32	+1.7	1
34	-0.9	3

\* Blood culture, *Pneumococcus* Type II colonies  $\infty$  per cc.

TABLE XIV.

*Change in Oxygen Capacity in Non-Fatal Cases.*

Case No.	Change in capacity per 100 cc. of blood.	Interval.	Remarks.
	<i>cc.</i>	<i>days</i>	
3	-0.0	7	
11	-2.1	16	
15	-1.1	8	
17	-2.0	21	
19	-2.1	9	No cyanosis.
23	+2.0	1	
24	+1.3	19	No cyanosis.
35	-4.2	24	
37	+0.2	1	No cyanosis.

*Oxygen Consumption and Heart Failure.*

Lundsgaard<sup>1</sup> pointed out that the oxygen consumption, *i.e.* the difference between the arterial and venous oxygen content, increases in cardiac insufficiency. It is also increased by exercise, and presumably by other factors, such as fever, which accelerate the metabolism, unless an equivalent acceleration in circulation occurs.



The oxygen consumption in the series of pneumonia cases presented here is from 0.7 to 10.5 cc. of oxygen per 100 cc. of blood, the average values ranging from 3 to 5 cc., which is the usual range in normal individuals. As a rule, the values for the oxygen consumption in the fatal cases or in the extremely sick were no greater than those in the non-fatal cases, or in the less acutely ill. This would indicate that in the types of pneumonia (chiefly post influenza) represented by our cases the cardiac output does not fall below that normal for the resting organism.

#### SUMMARY.

1. A simple method for arterial puncture is given which does no permanent injury to the artery. Arterial and venous punctures have been done on 33 cases of pneumonia and five normal subjects, and the blood thus obtained has been studied with reference to the oxygen capacity and arterial and venous unsaturation.

2. In five normal subjects the mean arterial unsaturation was 5 per cent of the total oxygen capacity; the mean venous unsaturation was 26.8 per cent.

3. In the pneumonia cases the arterial oxygen unsaturation varied over a wide range. The arterial unsaturation varied from 0.0 to 68.2 per cent, the venous from 14.4 to 85.5 per cent. In the fatal cases as opposed to the non-fatal cases of pneumonia, the mean arterial oxygen unsaturation was 32 per cent as against 13.9 per cent. As a rule, an arterial unsaturation of over 20 per cent was associated with a fatal outcome. Similarly, the mean venous oxygen unsaturation was 57 per cent in the fatal cases and 36.3 per cent in the non-fatal cases.

4. In five cases in which no cyanosis was observed at any time the mean arterial oxygen unsaturation was 5.4 per cent, the mean venous oxygen unsaturation 31.5 per cent. In cases which showed cyanosis of varying degree during the course of the illness, the mean arterial unsaturation was 24.7 per cent, and the mean venous unsaturation 44.5 per cent. Cases without cyanosis have an arterial unsaturation close to the normal.

5. There is a definite relation between the degree of cyanosis and the per cent of arterial unsaturation. With increasing cyanosis

the arterial unsaturation becomes greater. The venous unsaturation varies similarly.

6. In individual cases with marked cyanosis associated with high arterial unsaturation, the clinical improvement of the patient and the diminution of the cyanosis are accompanied by a similar diminution in the arterial and venous unsaturation. Conversely, an increase of cyanosis is accompanied by an increase in arterial unsaturation.

It is evident that the cyanosis of pneumonia patients is due to the incomplete saturation of venous blood with oxygen in the lungs, and that the various shades of blue observed in the distal parts are caused by an admixture of reduced hemoglobin and oxyhemoglobin in the superficial capillaries.

7. No unusually low total oxygen capacities were observed, even in fatal cases with intense cyanosis. On the contrary, in these cases the total oxygen capacity was unusually high, pointing toward a concentration of the blood. Again in only one case was there any marked fall in the oxygen capacity during the illness. Therefore, methemoglobin formation, in these cases, can hardly have occurred to such an extent as to be an important factor in the production of cyanosis. Of the 33 cases studied, however, only seven were lobar pneumonia, the rest being of types ordinarily unusual, which have accompanied the recent influenza epidemic; and of the seven, not all were in all respects typically lobar. The possibility still remains, therefore, that in typical lobar pneumonia caused by the pneumococcus methemoglobin may play a part in the cyanosis.

8. The oxygen consumption, *i.e.* difference between arterial and venous contents, was within normal limits, indicating that the cardiac output was not diminished in the cases (chiefly post influenza) of pneumonia studied.

#### EXPLANATION OF PLATES.

##### PLATE 13.

FIG. 1. Case 39. Arterial unsaturation 14.5 per cent. There is a moderate cyanosis which is confined to the finger-nails. The color is heliotrope.

FIG. 2. Case 25. In this case there is an intense (++++) cyanosis of the fingers associated with an arterial unsaturation of 44.1 per cent. Cyanosis extends as high as the terminal joint and then fades out imperceptibly.

## PLATE 14.

FIG. 3. Case 34. Bronchopneumonia; influenza. Arterial unsaturation 29.6 per cent. The entire face is diffusely cyanotic with no especial localization. The color is a leaden blue. The fingers in this case were markedly blue.

FIG. 4. Case 31. Multiple pulmonary abscesses (*Staphylococcus aureus*). Arterial unsaturation 15.3 per cent. There is a marked cyanosis of cheeks, nose, lips, and ears. On the cheek the color is a cherry-red-blue at the center, fading at the periphery to a heliotrope.

## PLATE 15.

FIG. 5. Case 35. Bronchopneumonia. Arterial unsaturation 33 per cent. There is a sharply localized area of cyanosis of the cheek. The rest of the face is relatively free. The fingers had a +++ cyanosis.

5-7/1



FIG. 1.



FIG. 2.

(Stadie: Oxygen of the blood in pneumonia.)







FIG. 3.



FIG. 4.

(Stadie: Oxygen of the blood in pneumonia.)





FIG. 5.

(Stadie: Oxygen of the blood in pneumonia.)





## STUDIES OF ACIDOSIS.

### XIV. DETERMINATIONS OF BICARBONATE IN THE BLOOD PLASMA OF DIFFERENT SPECIES BY THE TITRATION AND CARBON DIOXIDE CAPACITY METHODS.

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(Received for publication, July 9, 1919.)

In a previous paper<sup>1</sup> Van Slyke, Stillman, and Cullen have described a method for titrating the bicarbonate content of the blood serum or plasma, and given results obtained with human and rabbit plasmas. In the present paper we give a more extended comparison of results obtained by the titration method with parallel results by the carbon dioxide capacity method. We have used plasmas from the blood of man, dogs, sheep, rabbits, and chickens.

In each case the bicarbonate has been determined before and after the addition of known amounts of standard acetic acid, as in the last experiment on page 576 of the former paper.<sup>2</sup> 5 cc. portions of oxalate plasma were treated with water, or water plus 0.1 N acetic acid, making the total volume up to 6.5 cc. Portions were then taken for bicarbonate determinations by the carbon dioxide capacity method<sup>3</sup> and by the titration method. Neutral red was used as indicator.

In order to avoid the use of decimals, we have expressed the results in terms of millimolecular concentration (1 millimolecular = 0.001 molecular), rather than molecular.

In the first determination with each plasma no acid was added, but the 5 cc. of plasma were diluted to 6.5 cc. with water. Consequently in order to calculate the bicarbonate concentrations of

<sup>1</sup> Van Slyke, D. D., Stillman, E., and Cullen, G. E., *J. Biol. Chem.*, 1919, xxxviii, 167.

<sup>2</sup> Van Slyke, D. D., Stillman, E., and Cullen, G. E., *Studies from The Rockefeller Institute for Medical Research*, 1920, xxxiii, 569.

<sup>3</sup> Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 317.

the undiluted normal plasmas, the values given are to be multiplied by  $\frac{6.5}{5}$ , or by 1.3. The values so calculated agreed well with those obtained by direct determinations on undiluted plasma.

*Testing Standard 0.02 N NaOH for Carbonate.*—The importance of using carbonate-free standard alkali was emphasized in the former paper. The solutions should be made up as there described, using only boiled water, be kept in paraffin-lined bottles, and be protected from atmospheric  $\text{CO}_2$  by soda-lime tubes. They should be tested for carbonate as follows.

TABLE I.

*Bicarbonate Determinations in Dog Plasma.*

Plasma.		0.1 N acetic acid.	Water.	$\text{CO}_2$ bound as bicarbo- nate per 100 cc. of treated plasma.	Concentration of bicarbonate.		Fall in bicarbonate concentration.		Concen- tration of acetic acid added.
No.	Volume.				By $\text{CO}_2$ capacity.	By titra- tion.	By $\text{CO}_2$ capacity.	By titra- tion.	
	cc.	cc.	cc.	cc.	millimol.	millimol.	millimol.	millimol.	millimol.
I	5	0	1.5	41.4	18.5	16.6	0	0	0
	5	0.5	1.0	27.1	12.1	9.8	6.4	6.8	7.7
	5	1.0	0.5	2.6	1.2	3.2	15.3	13.4	15.4
II	5	0	1.5	48.1	21.5	20.7	0	0	0
	5	0.5	1.0	29.0	12.9	13.4	8.6	7.3	7.7
	5	1.0	0.5	6.1	2.7	5.8	18.8	14.9	15.4
III	5	0	1.5	38.5	17.2	15.1	0	0	0
	5	0.5	1.0	22.6	10.1	8.1	7.1	7.0	7.7
	5	1.0	0.5	8.5	3.7	1.2	13.5	13.9	15.4
IV	5	0	1.5	52.4	23.4	21.0	0	0	0
	5	0.5	1.0	34.0	15.1	13.8	8.3	7.2	7.7
	5	1.0	0.5	17.9	8.0	6.0	15.4	15.0	15.4

To 5 cc. of 0.02 N HCl in a 200 cc. round flask, add from a freshly filled burette about 4.8 cc. of the 0.02 N NaOH to be tested, with 0.3 cc. of neutral red solution. The mixture should be strongly acid to the indicator. The solution is rotated for 1 minute in the flask to permit the escape of  $\text{CO}_2$ , and is then transferred to a 50 cc. Erlenmeyer flask and titrated as in plasma analyses, the total amount of 0.02 N NaOH required to give the end-point being noted.

A duplicate titration is performed in the same way, except that

TABLE II.

*Bicarbonate Determinations in Chicken Plasma.*

Plasma.		0.1 N acetic acid.	Water.	CO <sub>2</sub> bound as bicarbo- nate per 100 cc. of treated plasma.	Concentration of bicarbonate.		Fall in bicarbonate concentration.		Concen- tration of acetic acid added.
No.	Volume.				By CO <sub>2</sub> capacity.	By titra- tion.	By CO <sub>2</sub> capacity.	By titra- tion.	
	cc.	cc.	cc.	cc.	millimol.	millimol.	millimol.	millimol.	millimol.
I	5	0	1.5	51.4	23.0	23.0	0	0	0
	5	0.5	1.0	32.8	14.6	15.6	8.4	7.4	7.7
II	5	0	1.5	39.9	17.8	18.2	0	0	0
	5	0.5	1.0	25.0	11.1	10.1	6.7	8.1	7.7
	5	1.0	0.5	7.5	3.3	3.2	14.5	15.0	15.4
III	5	0	1.5	33.4	14.9	14.1	0	0	0
	5	0.5	1.0	18.3	8.2	6.8	6.7	7.3	7.7
	5	1.0	0.5	0.0	0.0	0.8	14.9	13.3	15.4
IV	5	0	1.5	35.4	15.8	15.2	0	0	0
	5	0.5	1.0	20.8	9.8	8.5	6.0	6.7	7.7
	5	1.0	0.5	6.2	2.8	0.0	13.0	15.2	15.4

TABLE III.

*Bicarbonate Determinations in Sheep Plasma.*

Plasma.		0.1 N acetic acid.	Water.	CO <sub>2</sub> bound as bicarbo- nate per 100 cc. of treated plasma.	Concentration of bicarbonate.		Fall in bicarbonate concentration.		Concen- tration of acetic acid added.
No.	Volume.				By CO <sub>2</sub> capacity.	By titra- tion.	By CO <sub>2</sub> capacity.	By titra- tion.	
	cc.	cc.	cc.	cc.	millimol.	millimol.	millimol.	millimol.	millimol.
I	5	0.0	1.5	43.9	19.6	19.6	0	0	0
	5	0.5	1.0	25.9	11.5	11.7	8.1	7.9	7.7
	5	1.0	0.5	13.6	6.1	2.2	13.5	17.4	15.4
II	5	0.0	1.5	52.3	23.3	23.7	0	0	0
	5	0.5	1.0	35.4	15.8	16.6	7.5	7.1	7.7
	5	1.0	0.5	22.3	9.9	9.2	13.4	14.5	15.4
III	5	0.0	1.5	46.7	20.8	20.3	0	0	0
	5	0.5	1.0	28.9	12.9	11.6	7.9	8.7	7.7
	5	1.0	0.5	17.4	7.8	2.2	13.0	18.1	15.4
IV	5	0.0	1.5	42.9	19.6	17.7	0	0	0
	5	0.5	1.0	28.9	12.9	11.0	6.7	7.7	7.7
	5	1.0	0.5	14.8	6.6	1.8	13.0	15.9	15.4



there is no agitation to remove carbon dioxide, the 0.02 N HCl plus 20 cc. of water being placed directly in the 50 cc. Erlenmeyer flask, and the 0.02 N NaOH being added with a minimum of stirring.

If there is no carbonate in the standard NaOH solution the two titrations give identical results. The difference should preferably not exceed 0.1 cc., and if it exceeds 0.2 cc. the alkali should not be used.

TABLE IV.  
*Bicarbonate Determinations in Human Plasma.*

Plasma.		0.1 N acetic acid.	Water.	CO <sub>2</sub> bound as bicarbo- nate per 100 cc. of treated plasma.	Concentration of bicarbonate.		Fall in bicarbonate concentration.		Concen- tration of acetic acid added.
No.	Volume.				By CO <sub>2</sub> capacity.	By titra- tion.	By CO <sub>2</sub> capacity.	By titra- tion.	
	cc.	cc.	cc.	cc.	millimol.	millimol.	millimol.	millimol.	millimol.
I	5	0.0	1.5	51.8	23.1	23.9	0	0	0
	5	0.5	1.0	36.9	16.5	14.4	6.6	9.5	7.7
	5	1.0	0.5	21.8	9.7	8.5	13.4	15.4	15.4
	5	1.5	0.0	9.6	0.4	1.7	22.7	22.2	23.1
II	5	0.0	1.5	52.2	23.3	24.1	0	0	0
	5	0.5	1.0	38.9	17.4	16.4	5.9	7.7	7.7
	5	1.0	0.5	24.4	10.9	9.9	12.4	14.2	15.4
	5	1.5	0.0	9.6	1.7	1.9	21.6	22.2	23.1
III	5	0.0	1.5	51.4	22.9	23.6	0	0	0
	5	0.5	1.0	36.8	16.4	16.3	6.5	7.3	7.7
	5	1.0	0.5	21.9	9.8	9.4	13.1	14.2	15.4
	5	1.5	0.0	9.6	1.7	1.9	21.2	21.7	23.1
IV	5	0.0	1.5	53.6	23.9	24.6	0	0	0
	5	0.5	1.0	38.2	17.0	17.2	6.9	7.4	7.7
	5	1.0	0.5	23.5	10.5	10.8	13.4	13.8	15.4
	5	1.5	0.0	12.1	5.4	3.3	18.5	21.3	23.1

Tables I to IV show the parallel results obtained in determining the bicarbonate in the blood plasma of four different species by the titration and carbon dioxide capacity methods.

*Comparison of Results by the Two Methods.*—Results by titration agreed with those by CO<sub>2</sub> capacity as a rule within less than 2 millimolecular in plasma bicarbonate concentration, although in a few analyses this difference was exceeded. In the human plasmas the

average difference was 0.8 millimolecular, corresponding to 1.7 volumes per cent of plasma  $\text{CO}_2$ . The results confirm the conclusions reached in the former paper, that the two methods may be used interchangeably for clinical and for many experimental purposes.

Results of practically the same degree of accuracy were obtained with plasmas from the different species examined; viz., man, dog, rabbit, sheep, and chicken.

*Comparison of Amount of Added Acid with Fall in Bicarbonate Concentration.*—With both methods the amount of added acid was indicated by an approximately equivalent fall in plasma bicarbonate, the average difference between the calculated and observed changes being 1.4 millimolecular concentration by the  $\text{CO}_2$  capacity method and 0.9 millimolecular by the titration.



## THE STREPTOCOCCI OF EQUINES.\*

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PLATE 12.

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### INTRODUCTION.

Among the domestic animals no species is apparently more susceptible to infection with streptococci than the horse. In at least two diseases of the respiratory tract these microorganisms play a considerable part either as primary or secondary invaders. Streptococci are also responsible for many wound infections in horses and particularly in foals. Aside from a specific relationship to equine diseases, the relationship of the pathogenic streptococci found in horses to those of man is worthy of careful study.

Schütz (1) and Chantemesse and Delamotte (2) observed in the pneumonic areas of the lungs of horses suffering from influenza diplococci which when cultivated in bouillon developed as chains. Throughout the literature these streptococci are frequently referred to as the diplococcus or bicoccus of pneumonia.

Among the earlier workers Schütz (3), Baruchello (4), Sand and Jensen (5), and Poels (6) isolated or observed long chained streptococci in the nasal discharges and pharyngeal abscesses of horses suffering from strangles (adenitis equorum).

The earlier workers described the morphologic and cultural characters as they occurred on a few simple media such as agar, coagulated serum, and in bouillon and they attempted to show specific differences between these two streptococci. Their pathogenicity for mice and rabbits was clearly pointed out.

Galtier and Violet (7) also succeeded in isolating cocci in chains from the lungs of horses which had died of influenza. They observed that the length of chains varied widely in different tissues and cultures. In attempting to ascertain the source of the organism, they examined the nasal and pharyngeal mucosa

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\* This study was conducted during the war as a part of a study of infections occurring among horses, as a measure of military importance.



and feces of normal horses. They came to the conclusion that the specific streptococcus existed as a saprophyte in all these places. The characters on which the identifications were made were insufficient to separate streptococci into the various groups.

Lignières (8) isolated streptococci from the lungs, pleural fluid, liver, spleen, kidneys, and blood of horses dying of influenza. The cocci were usually ovoid and retained the stain by Gram's method. They grew as flocculi in bouillon and produced acid. Milk was usually coagulated in 24 to 48 hours. His cultures were pathogenic for mice and rabbits but guinea pigs were more resistant. 2 cc. of a bouillon culture injected subcutaneously into a horse produced an abscess. He compared Schütz's pneumonia diplococcus with Galtier and Violet's and his own streptococci and believed they were of the same species. He then undertook to show that Schütz's strangles streptococcus was identical with those isolated from influenza. Inasmuch as morphology and pathogenicity seemed to be the principal characters on which the comparisons were made, the results were not clear, although he regarded the streptococci as the same species. For a time Lignières was inclined to believe that streptococci were the cause of influenza but later he succeeded in isolating a rod-shaped organism of the septicemia hemorrhagica group which he claimed to be the agent. A horse inoculated with this culture died; not only was the bacterium recovered from the tissues but a streptococcus as well.

Ostertag (9) states that the nasal secretion of normal horses contains a mixture of bacteria including pus streptococci. He believed that these pus streptococci were identical with those found in influenza, but were not the cause of the disease since intranasal inoculation of material (nasal discharge) containing them failed to produce influenza.

During the next few years many writers discussed these organisms and their relationship to the diseases with which they were associated; in attempts to differentiate them, their virulence for mice and rabbits, the character of the growth in bouillon, and the length of chains proved as inconclusive as formerly. Thus Pfeiler (10) showed that the streptococci isolated from pyogenic conditions and pneumonia grew abundantly throughout bouillon. Bouillon cultures produce septicemia in mice. The strangles streptococcus grew more sparsely in bouillon and left the medium clear. Mice developed pyemia after subcutaneous injections. 50 to 60 cc. of a 24 hour bouillon culture of the pneumonia streptococcus injected intravenously into horses produced a severe reaction which usually terminated in pneumonia or pleuritis. In addition he found agglutinins for the streptococcus in the blood of horses suffering from influenza. He concluded that it was the cause of influenza.

Todd (11) described cocci occurring in chains of 30 to 60 individuals which he isolated from strangles abscesses. Microscopic examination of the nasal discharges usually revealed pairs and short chains. In bouillon they grew at the bottom of the tube, leaving the medium clear. Milk was coagulated in 6 or 8 days. Mice were exceedingly susceptible, rabbits more resistant. Horses when

inoculated subcutaneously with bouillon cultures developed abscesses. Cultures injected into the jugular vein caused only a slight general reaction. From his studies he does not believe that the causative streptococci are carried on the mucosa of the upper air passages of normal horses, but points out that they may be harbored for long periods after recovery.

Jensen (12) in discussing the specific prophylaxis and therapy of the streptococcic diseases of animals touches on the relationship of various streptococci in strangles, influenza, petechial fever, and wound infections. He considers that Holth (13) described a streptococcus (*Streptococcus equi*) which is the cause of strangles. It produces acid in dextrose, maltose, saccharose, and salicin but fails to ferment lactose, raffinose, inulin, or mannite, thus differing sharply from the diplococcus of influenza (*Brustseuchekokkus*) and the pyogenic streptococci. Jensen considers the etiology of influenza still in doubt but believes that the diplococci associated with it belongs to the *Streptococcus pyogenes* group. They differ from *Streptococcus equi* in their ability to ferment lactose and sorbite (Holth).

Bemelmans (14) brings forth a clinical observation to support the claim that strangles and influenza are not due to the same streptococcus. Thus cavalry horses that had recovered from strangles 9, 11, and 45 days previously came down with influenza. He argues that if the infections were caused by the same streptococcus the horses would not have been susceptible to a second attack within such a short period. Bemelmans also quotes Holth, who showed that the strangles organism was an encapsulated streptococcus which fermented dextrose, saccharose, maltose, and salicin but failed to break down lactose, raffinose, inulin, or glycerol.

Koch and Pokschischewsky (15) compared the human streptococcus, *erysipelatos*, with various strains of horse streptococci. The human types had been isolated by the writers. The horse streptococci, with the exception of three strains, had been isolated from cases of strangles in other laboratories. They had been grown on artificial media for long periods (1 to 5 years). The details concerning the more important biological characters are given in twenty instances. All the human strains fermented dextrose, saccharose, maltose, and mannite. Three failed to ferment lactose; the others acted upon this substance. All the horse strains produced acid in dextrose, saccharose, and maltose. Raffinose and mannite were not acted upon. Five strains fermented lactose; the others failed to do so. Both the human and equine streptococci produced hemolysis in blood agar plates. The equine types had a tendency to produce larger areas of hemolysis. The net acid production after several days incubation when measured with 0.05 N sodium hydroxide with phenolphthalein as indicator failed to show marked differences between the horse and human strains. Four strains of each type when grown in dextrose and titrated after maximum acid production had been reached averaged for the human +2.55 per cent and the equine +2.65. After studying the relative virulence of each type they concluded that the streptococcus of strangles is closely related to human *Streptococcus erysipelatos*. It is

interesting to observe that they assumed that the horse streptococci were all of one species whereas in reality there were two, one capable of fermenting lactose and the other unable to do so. The same holds true with the human types. The mannite fermentation by all the human strains was not regarded as of particular significance.

To Schofield (16) we are indebted for a clear description of the streptococci occurring in septic arthritis of foals in Canada. In fifteen cases he found streptococci in the affected joints. The cocci are described as growing in pairs on media other than bouillon. They stain by Gram's method. To determine hemolysis defibrinated rabbit blood, 1 cc., and agar, 10 cc., were poured into Petri dishes and allowed to congeal; the surface was then streaked with culture. Type II produced a clear space 5 to 6 mm. wide about the growth. The clear area extended to the bottom of the dish. In Type III he found that hemolysis took place immediately surrounding the growth but left a discolored zone between the hemolyzed area and the surrounding medium. Apparently the only differences between Types II and III were the size of the hemolytic area and the pathogenicity for rabbits. Type I is spoken of as the Schütz streptococcus but, since his protocols fail to show its presence in the joints or give any data concerning it and since no description of it is offered, it might as well have been omitted. Both Types II and III ferment dextrose, lactose, saccharose, and salicin but fail to ferment inulin, raffinose, dulcitol, and mannitol. He states: "The quantity of acid produced varied with the same strains under different conditions and with similar strains under the same conditions." It is pointed out, however, that no strain lost or acquired fermentative power.

Mathers (17) cultivated streptococci from the nasal discharges of horses suffering from influenza. In seven of twenty-two blood cultures streptococci developed. In addition he found them in exudate from the eye, the pleura, in purulent joints, and involved lymph nodes. Hemolytic streptococci were the only organisms occurring with any degree of regularity. On standard blood agar plates (1 cc. of defibrinated goat blood and 9 cc. of agar) the colonies were round, moist, and adherent. They were surrounded by a clear area of hemolysis 2 to 4 mm. in diameter. The morphology varied; pairs, chains of pairs, and chains were recorded. Many were Gram-negative. They fermented dextrose, lactose, saccharose, and salicin. Milk was acidulated but not coagulated. It is stated that many variations in the fermentative characters occurred. The streptococci were not highly pathogenic for rabbits but highly virulent for horses. Intranasal inoculation of a normal horse with "extracts" prepared from the nasal discharge of a horse suffering from influenza resulted in a "typical attack" of the disease. To test Gaffky and Lührs' (18) findings that influenza is caused by a filterable agent, a series of sixteen inoculations was made. The nasal exudate from diseased horses was suspended in salt solution and extracts of the pneumonic lung were prepared and both filtered. In no instance was influenza produced by the injection of filtrates. Intranasal sprays with the sediment obtained from 180 cc. of an ascitic dextrose broth culture produced a severe rhinitis



and pleuritis accompanied with fever and rapid pulse and respiration. The horse recovered in 18 days. Another horse was injected intravenously with sediment from the same amount of culture. It developed symptoms of influenza and died 8 days later. Autopsy revealed fibrinous pleuritis, bronchopneumonia, and evidences of septicemia.

Since the relationship of the strangles streptococcus with those associated with influenza seemed confused, it was considered advisable to study these streptococci with the methods now in use. In addition, the problem of ascertaining whether streptococci similar to those found in strangles and influenza were carried on the nasal mucosa or in the pharynx of horses presented itself. Studies were made on the following classes of horses: (1) normal horses (eastern) that had been in this vicinity for some time; (2) apparently normal horses that had recently been shipped from the West; (3) those suffering from strangles, influenza, rhinitis, and purulent conditions.

*Streptococci of the Nasal Mucosa and Upper Pharynx of Normal Horses.*

Sterile cotton swabs wound on bale wire, 15 and 30 cm. in length, were employed to obtain material. The shorter were used for the nasal passages and the longer for the pharynx. The same swab was used in both nostrils. In making preparations from the throat the mouth was held open with a speculum, the tongue depressed, and the swab brushed over the mucosa of the upper pharynx. Within 2 hours the swabs were agitated for a few seconds in tubes containing 10 cc. of sterile 0.9 per cent salt solution. Two or three loopfuls of suspension were inoculated into a tube containing 12 cc. of melted veal infusion agar (2 per cent) cooled to 45°C. and the whole was plated with 1 cc. of defibrinated horse blood. After 24 hours incubation at 38°C. subcultures were made from colonies resembling streptococci.

The results of the examinations of the thirty eastern horses may be summarized as follows:

Horses carrying non-hemolytic streptococci on the nasal mucosa . . . .	8
Horses carrying hemolytic streptococci on the nasal mucosa . . . . .	0
Horses carrying non-hemolytic streptococci in the pharynx . . . . .	6
Horses carrying hemolytic streptococci in the pharynx . . . . .	18



The morphological and cultural characters of these streptococci are given in Tables I, II, and III. Inoculations from 18 hour broth cultures were made into tubes containing 13 cc. of fermented bouillon adjusted to +0.6 to +0.8 (phenolphthalein), to which had been added sufficient amounts of the carbohydrates and other substances to make a 1 per cent solution. Titrations were made after 5 days incubation at 38°C. An incubation period of this length was considered sufficient since the maximum acid production is usually reached within 36 hours. The amounts recorded in the tables represent the net acid production.<sup>1</sup> Litmus milk was likewise incubated for 5 days. On removal from the incubator if not coagulated the tubes were boiled for a few minutes. The figures under the column "Diameter of area of hemolysis" represent the diameter of hemolysis about the deep colonies after 24 hours incubation.

Smith and Brown (19) and Brown (20) describe two principal types of hemolysis. The alpha type is characterized by a partial hemolysis and greenish discoloration of the red cells immediately surrounding the deep colonies. This zone is succeeded by another clear band of partially hemolyzed cells. In the beta type the hemolytic area is sharply defined and clear, since the cells are completely hemolyzed. The beta type has been frequently observed among the horse streptococci but the alpha type has not been met with. A characteristic hemolytic zone has been noted with considerable frequency in the throat strains from horses. It surrounds both the surface and deep colonies as a clearly defined area varying in color from light orange to a delicate red. In certain instances there is a narrow clearer line between the colony and the larger discolored area. Microscopic examination reveals the presence of aggregates of unhemolyzed red cells throughout the lighter colored zone. The intensity of color appears to depend on the number of unhemolyzed cells. If the incubated plates are stored for 24 or 48 hours the color of the unhemolyzed cells has a tendency to deepen. When rabbit blood is used in the plate cultures the picture is not so striking. Colonies surrounded by the narrow clear line have been indicated

<sup>1</sup> The total acidity reached in the various test substances may be approximated by adding 0.7 per cent to the amounts given in the table.

in the tables as Type xa (Figs. 1 and 2), and those which showed only the partially hemolyzed area as Type x (Figs. 3 and 4).

Since all strains were Gram-positive, mention of this characteristic has been omitted from the tables.

The nasal strains fall into two groups when classified on the basis of lactose fermentation. In both the proportion of mannite-fermenting strains is high. The predominating type is a streptococcus which produces acid in dextrose, lactose, saccharose, maltose, man-

TABLE I.

*Non-Hemolytic Streptococci from the Nasal Mucosa of Eastern Horses.*

Strain No.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.							
				Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
				per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
N 5	L. C.*	Clear.	Coagulated on boiling.	3.0	2.6	2.4	2.4	0.0	0.0	2.6	1.9
N 8	"	"	" " "	3.4	2.8	2.8	2.5	0.0	0.0	2.5	2.6
N 9	"	Turbid.	" " "	2.7	2.8	2.8	2.4	0.0	0.0	1.9	2.1
N 10	"	"	" " "	2.5	2.3	2.4	2.2	0.1	0.2	1.9	2.4
N 11	"	Clear.	" " "	2.3	2.1	0.0	2.2	0.0	0.0	1.7	2.3
N 13	"	"	" " "	2.5	2.4	2.3	2.4	0.2	0.0	2.7	2.0
N 15	"	"	" " "	3.2	3.5	2.2	3.0	0.2	0.1	2.7	2.5
N 19	"	"	" " "	3.5	3.5	3.5	3.0	0.0	0.0	2.5	2.3
N 6	Pairs and S. C.	Turbid.	Unchanged.	1.7	0.2	1.4	1.5	0.2	0.2	0.2	1.6
N 16	" " "	"	"	1.8	0.0	1.8	1.8	0.1	0.0	0.0	1.9
N 3	L. C.	Clear.	"	3.0	0.1	2.7	3.0	2.8	0.1	2.7	3.2
N 4	"	"	"	2.4	0.1	2.4	2.4	0.1	0.1	2.5	2.4

\* L. C. indicates chains of 20 or more elements; threads of 8 to 20 are indicated as M. C., and those made up of 6 or 8 cocci as S. C.

nite, and salicin. One strain failed to ferment saccharose. None of these streptococci proved pathogenic for mice when 0.1 cc. of a 24 hour bouillon culture was injected into the peritoneal cavity.

Six of the seven strains of non-hemolytic streptococci from the pharynx failed to ferment lactose. They differed from the nasal types in their inability to ferment mannite. All except one attacked raffinose or inulin. The hemolytic streptococci possessed similar fermentative characters, although the proportion of lactose-fermenting

and non-lactose-fermenting strains was nearly equal. They too were capable of breaking down raffinose or inulin. Acid production in mannite was not infrequent but always occurred in combination with raffinose or inulin fermentation. Strain P 14 differed sharply from the others in its cultural characters and its pathogenic properties. This species was extremely virulent for mice and rabbits. The others failed to produce ill effects when 0.1 cc. of a 24 hour bouillon culture was injected into the peritoneal cavity of mice, or when 1 cc. was injected intravenously into rabbits.

TABLE II.

*Non-Hemolytic Streptococci from the Pharynx of Eastern Horses.*

Strain No.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.							
				Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
				per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
P 41	Pairs and S. C.	Turbid.	Unchanged.	4.3	0.0	3.7	4.0	3.5	3.4	0.0	3.1
P 45	L. C.	"	"	3.0	0.1	2.4	2.6	2.5	3.0	0.2	3.0
P 13	Pairs.	Clear.	"	3.8	0.1	2.6	3.0	0.3	2.6	0.1	2.7
P 42	L. C.	Turbid.	"	4.8	0.0	4.4	4.5	0.0	4.7	0.1	4.8
P 52	"	"	"	3.2	0.1	3.0	2.9	0.1	2.7	0.1	2.8
P 5	M. C.	"	"	4.7	0.1	4.5	3.2	0.1	0.2	0.1	3.9
P 36	"	"	Coagulated on boiling.	3.4	2.9	3.5	3.6	2.5	3.6	0.1	3.5

From these studies several points stand out clearly. The bulk of the nasal flora is made up of non-hemolytic streptococci which ferment mannite. In the pharynx both hemolytic and non-hemolytic streptococci which ferment raffinose and inulin predominate. Strains which fail to produce acid in lactose are frequent in both regions.

Since the preceding results had established sufficiently the streptococcic flora of normal horses which had been in the East for some time, studies were begun on horses which had been recently shipped from the West. These animals were apparently normal when the cultures were made. Horses were examined over a period of 5 months. It may be assumed that they had been exposed to influenza and other diseases at various shipping points. Some of their fellows were

TABLE III.

*Hemolytic Streptococci from the Pharynx of Eastern Horses.*

Strain No.	Group- ing.	Growth in bouillon.	Litmus milk.	Production of acid in.								Diameter of area of hemol- ysis.  mm.	Type of hemol- ysis.
				Dextrose.	Lactose.	Saccharose.	Maltose.	Rafinose.	Inulin.	Mannite.	Salicin.		
				per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent		
P 3	L. C.	Clear.	Unchanged.	2.1	0.0	1.9	2.0	1.7	1.9	0.0	2.0	3.0-3.5	Beta.
P 16	"	"	"	2.7	0.0	2.8	2.4	2.8	0.0	3.3	4.0-5.0	x	
P 19	"	Turbid.	"	2.8	0.1	2.5	2.8	2.3	3.2	0.0	2.8	3.0	x
P 20	"	Clear.	"	1.3	0.0	1.4	1.5	1.9	1.3	0.0	1.8	3.5-4.0	Beta.
P 30	"	"	"	2.8	0.0	2.6	2.6	2.2	1.7	0.0	2.2	1.0	x
P 39	M. C.	"	"	2.8	0.0	2.2	2.1	2.2	1.8	0.0	0.6	2.5-3.0	x
P 43	Pairs and S. C.	Turbid.	"	4.0	0.0	3.7	4.0	3.5	3.4	0.0	0.8	3.5-4.0	x
P 11	" " "	"	"	3.7	0.3	3.7	3.5	2.9	1.7	3.3	3.3	2.0-2.5	x
P 24	M. C.	"	"	2.6	0.0	2.3	2.6	0.0	2.2	0.0	2.4	2.5-3.0	Beta.
P 47	Pairs and S. C.	Clear.	"	2.4	0.0	2.4	2.2	0.0	2.4	0.0	2.4	2.5-3.0	x
P 26	S. C.	Turbid.	"	4.6	0.0	4.2	4.0	0.0	4.3	2.2	4.2	2.0	x
P 22	L. C.	Clear.	Coagulated on boiling.	1.7	1.3	1.7	1.8	1.4	1.8	0.0	1.8	3.0-4.0	x
P 35	"	"	" " "	3.8	3.5	3.8	3.5	0.6	3.7	0.2	4.0	3.0-3.5	x
P 38	M. C.	"	" " "	2.9	2.7	2.9	2.7	2.5	2.1	0.0	3.0	2.5-3.0	Beta.
P 51	L. C.	"	" " "	3.6	2.1	3.4	3.1	2.7	2.9	0.0	2.6	4.5-5.0	x
P 23	Pairs and S. C.	Turbid.	Acid.	2.8	2.6	3.0	3.1	2.5	2.3	2.8	2.9	2.5-3.0	Beta.
P 1	Pairs.	"	Coagulated on boiling.	3.4	3.1	3.2	3.3	2.9	0.0	3.1	2.9	4.0-5.0	xa
P 6	" and S. C.	"	" " "	3.1	2.7	3.1	3.2	2.6	0.0	2.6	2.9	4.0-5.0	xa
P 53	" " "	"	Acid.	3.8	3.2	3.7	3.8	3.2	0.0	2.9	3.8	3.0-3.5	x
P 40	M. C.	"	Firmly coag- ulated.	4.7	4.5	5.2	5.2	4.2	0.0	2.0	4.1	6.0-7.0	xa
P 14	"	Clear.	Coagulated on boiling.	3.4	2.8	3.1	3.3	0.0	0.0	0.0	2.4	3.0-3.5	Beta.



suffering from influenza when the cultures were made. In all probability many had been exposed repeatedly. It was impossible to observe them after they had left the sales stables; some may have come down later.

The following summarizes the results of the nasal and pharyngeal examinations of the twenty-three western horses:

Horses carrying non-hemolytic streptococci on the nasal mucosa . . . .	8
Horses carrying hemolytic streptococci on the nasal mucosa . . . . .	8
Horses carrying non-hemolytic streptococci in the pharynx . . . . .	11
Horses carrying hemolytic streptococci in the pharynx . . . . .	11

TABLE IV.

*Non-Hemolytic Streptococci from the Nasal Mucosa of Western Horses.*

Strain No.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.							
				Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
				per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
N 26	L. C.	Turbid.	Coagulated, not firmly.	4.1	3.0	3.9	3.6	0.0	0.0	2.7	3.5
N 40	"	Clear.	" on boiling.	3.5	3.7	3.7	3.3	0.0	0.2	2.7	2.6
N 44	"	"	" " "	2.9	2.5	3.4	3.1	0.0	0.0	2.9	2.5
N 45	"	"	" " "	3.9	3.2	3.0	3.4	0.1	0.1	3.0	4.1
N 27	Pairs and S. C.	Turbid.	" " "	3.2	3.0	3.1	3.2	0.0	0.3	2.0	0.0
N 41	" " "	"	" " "	3.6	3.4	3.8	3.8	0.1	3.3	0.1	0.0
N 24	M. C.	"	" " "	3.7	3.1	3.7	3.5	0.0	0.0	0.0	3.0
N 33	"	Clear.	Unchanged.	3.3	3.0	3.4	3.4	2.7	3.1	0.0	3.2
N 46	"	"	"	4.2	0.1	3.9	3.6	3.4	0.0	3.0	5.0

An outstanding feature is the presence of hemolytic streptococci in the lower nasal passages of 30 per cent of the western horses. Streptococci of this type were not found in the same region in the eastern horses. The morphological and cultural characters are given in Tables IV to VII.

It is interesting to observe that the non-hemolytic streptococci from the nasal mucosa are similar in both eastern and western horses. Mannite fermenters make up the majority of the flora, although raffinose and inulin fermentation was observed more frequently. A striking difference in the flora of the two classes of horses is the pres-

TABLE V.

*Hemolytic Streptococci from the Nasal Mucosa of Western Horses.*

Strain No.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.								Diameter of area of hemolysis.	Type of hemolysis.
				Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.		
				per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	mm.	
N 20	M. C.	Turbid.	Coagulated on boiling.	3.8	3.0	3.6	3.6	0.0	0.0	0.0	3.4	3.5-4.0	Beta.
N 25	L. C.	"	" " "	4.2	4.0	4.4	4.3	0.1	0.0	0.0	3.4	3.0-3.5	"
N 28	"	"	" " "	4.3	3.8	4.5	4.2	0.0	0.2	0.0	3.0	2.5-3.0	"
N 29	"	Clear.	" " "	4.6	3.2	4.2	4.2	0.0	0.0	0.0	3.4	3.5-4.0	"
N 38	M. C.	Turbid.	" " "	4.2	3.7	3.8	4.7	0.0	0.1	0.1	3.8	3.5-4.0	"
N 42	"	"	" " "	4.8	3.8	4.0	3.8	0.0	0.0	0.0	4.4	3.0	"
N 47	S. C.	Clear.	" " "	4.2	3.6	4.1	4.1	0.0	0.0	0.0	3.6	3.5-4.0	"
N 48	M. C.	"	" " "	4.1	3.6	4.0	4.0	0.2	0.2	0.0	3.5	3.5-4.0	"

TABLE VI.

*Non-Hemolytic Streptococci from the Pharynx of Western Horses.*

Strain No.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.							
				Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
				per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
P 48	L. C.	Turbid.	Unchanged.	3.5	0.0	3.0	3.3	3.0	3.2	0.2	3.1
P 67	S. C.	"	"	2.7	0.1	2.6	2.7	3.1	4.0	0.0	3.6
P 73	L. C.	"	"	3.9	0.0	3.8	4.0	3.2	3.4	0.0	3.3
P 74	"	"	"	4.2	0.0	3.7	3.8	3.5	4.3	0.1	3.9
P 84	S. C.	"	"	4.0	0.0	4.2	4.2	3.5	3.3	4.0	4.0
P 64	L. C.	Clear.	"	7.2	0.0	6.4	5.7	5.6	6.0	0.0	5.4
P 75	"	Turbid.	"	3.8	0.0	4.0	4.1	0.0	3.7	0.0	3.5
P 76	M. C.	"	"	3.3	0.2	3.4	3.1	0.0	3.3	0.0	2.7
P 58	Pairs and S. C.	"	Firmly coagulated.	4.4	3.6	3.5	3.4	3.3	2.9	0.0	3.0
P 60	L. C.	"	Coagulated on boiling.	3.6	3.0	3.6	3.3	3.5	3.7	0.0	3.6
P 69	M. C.	"	" " "	2.9	2.3	3.3	2.9	2.2	2.8	0.0	1.7
P 72	L. C.	"	" " "	3.8	3.0	3.9	3.4	3.4	3.2	0.0	3.7
P 78	"	Clear.	" not firmly.	4.1	3.5	3.1	3.8	2.9	4.1	0.0	3.2
P 83	S. C.	Turbid.	" on boiling	4.1	3.2	4.0	3.9	3.4	3.8	0.1	3.6

ence of pathogenic hemolytic streptococci (*Streptococcus pyogenes*) on the nasal mucosa of the western horses (Table V). These streptococci produced acid in dextrose, lactose, saccharose, maltose, salicin, and milk. The other substances were not fermented. Mice, when injected intraperitoneally with 0.1 cc. of a 24 hour bouillon culture, developed septicemia and died within 24 or 48 hours. Rabbits when injected intravenously with 1 cc. of culture were much more resistant;

TABLE VII.

*Hemolytic Streptococci from the Pharynx of Western Horses.*

Strain No.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.								Diameter of area of hemolysis.	Type of hemolysis.
				Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.		
				per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	mm.	
P 55	L. C.	Clear.	Coagulated on boiling.	4.0	3.3	3.9	3.6	0.1	0.0	0.0	4.0	3.0-3.5	Beta.
P 56	S. C.	"	Acid.	3.8	3.4	4.0	4.0	0.0	0.0	0.0	3.4	3.0-3.5	"
P 61	L. C.	Turbid.	Coagulated on boiling.	4.2	3.3	4.0	4.4	0.0	0.1	0.0	4.2	2.5-3.0	"
P 62	"	Clear.	" " "	4.4	3.6	4.1	4.5	0.1	0.2	0.0	3.6	3.0-3.5	"
P 63	M. C.	"	" " "	4.2	3.0	3.9	4.3	0.0	0.0	0.0	4.1	3.0-3.5	"
P 79	L. C.	Turbid.	Coagulated, not firmly.	4.6	3.9	4.3	4.5	0.1	0.0	0.0	5.0	3.0-3.5	"
P 80	S. C.	Clear.	" " "	5.3	4.3	4.9	5.3	0.0	0.0	0.0	4.6	3.5-4.0	"
P 81	L. C.	"	" " "	5.3	3.9	5.1	5.3	0.0	0.1	0.1	4.9	3.5-4.0	"
P 57	S. C.	Turbid.	Unchanged.	3.9	0.0	3.9	4.0	3.3	3.3	3.6	3.7	3.5-4.0	x
P 68	"	"	"	3.8	0.0	4.0	4.2	3.0	2.8	2.9	3.7	6.0-7.0	xa
P 66	L. C.	Clear.	"	2.0	0.0	1.8	1.9	2.4	2.0	0.0	1.7	2.5-3.0	xa
P 82	M. C.	"	Coagulated on boiling.	5.0	3.9	4.2	4.4	0.0	5.5	2.6	3.7	3.0-3.5	Beta.
P 65	L. C.	Turbid.	" " "	5.2	3.8	6.0	5.2	4.0	0.0	0.0	4.1	3.5-4.0	"

many became emaciated and showed severe temperature reactions. Some recovered while others developed purulent arthritis. Larger doses of culture usually produced septicemia.

The same groups which were encountered in the pharyngeal flora of eastern horses were observed in the western horses. Among the non-hemolytic streptococci raffinose- and inulin-fermenting types predominated. Strains which failed to ferment lactose were frequent.

The horses from the West carried *Streptococcus pyogenes* in the throat in eight instances. The other five strains given in Table VII are raffinose or inulin fermenters which may or may not act upon mannite. They are similar to those observed in Table III. Three streptococci of the *pyogenes* type from the pharynx possessed the same pathogenic properties for rabbits and mice as those found on the nasal mucosa.

*Pathogenic Streptococci of the Nasal Mucosa and Upper Pharynx of  
Diseased Horses.*

Pathogenic streptococci have been isolated from horses suffering from acute diseases of the respiratory tract (influenza, rhinitis, pharyngitis, and strangles) and from infected wounds. The material was obtained by brushing the affected surfaces with a sterile swab. Salt solution suspensions were then made. Plates were prepared within a few hours.

Among twenty-four cases of influenza hemolytic streptococci were found in considerable numbers in the nasal discharge from twenty-two. The plate cultures from the pharynx were positive in eight cases. When conjunctivitis (pink eye) was an accompanying factor streptococci were obtained in practically pure culture from the ocular exudate. It was possible to obtain material from six cases of strangles. Pus from various acute and chronic purulent conditions was suspended in salt solution from which plate cultures were made. Streptococci were frequently found in pure culture. Table VIII indicates the cultural characteristics of these streptococci.

It will be observed that the streptococci fall into two broad groups. The larger group, composed of streptococci from influenza, strangles (three cases), and all but one abscess are of *Streptococcus pyogenes* type. They produce acid in dextrose, lactose, saccharose, maltose, and milk but fail to acidulate raffinose, inulin, or mannite. The smaller group, *Streptococcus equi*, includes the non-lactose-fermenting strains which do not produce acid in raffinose, inulin, mannite, or milk. These were found in influenza and strangles, rhinitis and pharyngitis, and once in an abscess.

Both types are especially virulent for mice. Rabbits are more resistant. 1 cc. of a 24 hour bouillon culture of certain strains may



TABLE VIII.  
*Pathogenic Streptococci.*

Strain No.	Source.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.								Diameter of area of hemolysis.	
					Production of acid in.									
					Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.		
			per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	mm.	
In. 14	Nasal exudate; influenza.	L. C.	Clear.	Coagulated on boiling.	3.5	2.8	3.5	3.6	0.0	0.0	0.0	0.3	5.3	5-4.0
In. 15	"	"	"	"	3.7	2.9	3.8	3.6	0.0	1.0	0.3	7.3	0.3-5	
In. 19	"	"	Turbid.	"	4.2	3.3	3.9	4.4	1.0	0.0	0.4	3.0	3-5	
In. 20	"	Pairs and S. C.	Clear.	"	4.0	3.4	4.2	4.1	0.0	0.0	0.3	9.4	5-5.0	
In. 22	Pharynx;	L. C.	"	"	3.9	3.7	3.5	3.7	0.0	0.0	0.3	9.3	5-4.0	
In. 25	Nasal exudate;	"	"	"	5.0	3.8	4.7	4.2	0.0	0.0	0.4	3.0	3-5	
In. 26	"	"	"	"	4.1	3.0	4.0	3.8	0.0	0.0	0.3	5.3	0-3.5	
In. 31	"	"	Turbid.	"	4.0	3.4	4.1	4.0	0.0	0.0	0.3	6.3	5-4.0	
In. 32	"	"	"	"	5.0	4.1	4.7	5.0	0.0	0.0	1.4	1.3	5-4.0	
In. 36	Pharynx;	M. C.	"	"	4.3	3.5	3.9	4.2	0.0	0.0	0.3	4.3	0-3.5	
In. 38	Nasal exudate;	S. C.	Clear.	"	4.2	3.5	4.2	4.4	0.0	0.0	0.4	3.0	3-5	
In. 42	Exudate from eye;	M. C.	"	"	4.2	3.1	4.1	4.4	0.0	0.0	0.3	3.2	5-3.0	
In. 43	"	"	Turbid.	"	4.6	3.6	4.2	4.8	0.1	0.0	2.4	1.2	5-3.0	
In. 46	Nasal exudate;	"	Clear.	"	4.1	3.1	4.0	4.0	0.0	1.0	0.3	4.2	5-3.0	
In. 47	"	S. C.	"	"	4.3	3.7	4.1	4.0	0.0	0.0	0.3	4.2	5-3.0	
In. 48	Pharynx;	Pairs and S. C.	Turbid.	"	4.2	3.1	3.7	4.2	0.0	0.0	1.3	0.3	0-3.5	
In. 49	Nasal exudate;	M. C.	"	"	4.2	2.9	4.0	4.3	0.0	0.0	0.4	0.3	5-4.0	
In. 50	"	S. C.	"	"	4.3	2.9	3.7	4.3	0.0	0.0	0.4	0.3	5-4.0	
In. 52	Pharynx;	M. C.	"	"	4.1	2.8	4.1	4.0	0.0	1.0	0.3	8.4	0-4.5	
In. 53	"	Pairs and S. C.	"	"	4.3	3.3	3.8	4.3	0.1	0.0	0.3	9.3	0-3.5	

In. 54	Nasal exudate; influenza.	M. C.	Turbid.	Coagulated on boiling.	4.1	3.1	3.6	4.2	0.0	0.1	0.0	3.4	3.5	4.0
In. 55	"	L. C.	Clear.	"	4.3	3.6	4.2	4.2	0.0	0.0	0.0	3.5	3.5	4.0
In. 57	"	Pairs and S. C.	Turbid.	"	4.4	3.0	4.1	4.0	0.0	0.0	0.0	4.0	3.0	3.5
In. 58	"	"	"	"	4.6	3.0	4.2	4.3	0.0	0.0	0.0	3.8	3.3	4.0
In. 59	"	"	"	"	4.2	3.4	4.1	4.3	0.0	0.0	0.0	4.0	3.0	3.5
In. 60	"	S. C.	Clear.	"	4.1	3.0	3.7	3.9	0.0	0.0	0.0	3.2	2.0	2.5
In. 62	"	M. C.	Turbid.	"	3.7	3.3	3.5	3.5	0.0	0.0	0.0	3.2	3.0	3.5
In. 63	"	"	"	"	3.7	2.9	3.7	3.4	0.0	0.0	0.0	3.2	3.0	3.5
In. 64	"	L. C.	"	Partially coagulated on boiling.	4.0	3.1	3.0	4.1	0.0	0.0	0.0	3.2	3.0	3.5
In. 66	"	"	"	Acid.	3.9	3.1	3.8	3.9	0.0	0.0	0.0	3.4	2.0	2.5
In. 69	Pharynx;	"	"	Coagulated on boiling.	4.0	3.7	4.1	3.5	0.0	0.0	0.0	3.6	2.0	2.5
In. 70	"	"	"	"	3.2	3.2	3.1	3.8	0.0	0.0	0.0	1.3	3.2	0.2
In. 71	Nasal exudate;	"	Clear.	"	4.0	3.4	4.1	4.0	0.0	0.0	0.0	3.7	2.0	2.5
In. 76	"	S. C.	Turbid.	"	3.2	3.0	3.0	3.1	0.0	0.0	0.0	1.2	2.5	3.0
In. 77	Pharynx;	M. C.	Clear.	"	4.1	3.6	3.3	3.9	0.0	0.0	0.0	1.3	4.3	0.3
In. 79	Nasal exudate;	"	"	"	4.4	3.6	4.1	3.8	0.0	0.0	0.0	1.3	0.2	5.3
In. 81	"	"	Turbid.	"	3.7	2.7	3.3	3.7	0.0	0.0	0.0	1.3	2.2	5.3
In. 84	"	L. C.	Clear.	"	3.7	2.9	3.6	3.6	0.0	0.0	0.0	1.0	2.9	3.5
In. 88	"	"	Turbid.	"	3.1	2.9	3.1	3.4	0.0	0.0	0.0	1.0	2.3	2.5
In. 90	"	M. C.	"	"	3.8	3.1	3.6	3.5	0.0	0.0	0.0	2.7	2.5	3.0
Str. 1	"	"	"	"	4.4	3.5	4.1	4.2	0.0	0.0	0.0	1.0	3.8	3.0
Str. 20	"	"	"	Acid.	3.6	3.2	3.4	3.8	0.0	0.0	0.0	3.5	3.0	3.5
Str. 22	Abscess;	L. C.	Clear.	Coagulated on boiling.	3.7	2.5	3.3	3.5	0.0	0.0	0.0	3.4	3.0	3.5
HA 2	"	S. C.	"	"	3.7	2.9	3.7	3.6	0.0	0.0	0.0	3.1	2.5	3.0
HA 3	"	M. C.	Turbid.	"	4.2	3.3	3.8	3.3	0.0	0.0	0.0	1.0	4.1	3.5
HA 5	"	"	"	"	4.5	3.5	3.8	4.3	0.0	0.0	0.0	3.5	3.0	3.5
HA 6	"	S. C.	"	"	4.6	3.7	3.9	4.3	0.0	0.0	0.0	1.0	3.6	3.5
HA 7	"	M. C.	Clear.	"	4.3	3.3	3.6	4.1	0.0	0.0	0.0	3.9	3.5	4.0
HA 10	"	S. C.	Turbid.	"	4.9	3.7	4.6	4.4	0.0	0.0	0.0	3.5	2.0	2.5
HA 13	"	L. C.	Clear.	"	3.3	3.0	3.4	3.3	0.0	0.0	0.0	3.1	1.3	5.4

TABLE VIII—*Concluded.*

Strain No.	Source.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.						Diameter of area of hemolysis.	
					Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.		Mannite.
					per cent	per cent	per cent	per cent	per cent	per cent	per cent	mm.
IIA 16	Pl centa; abortion.	M. C.	Turbid.	Coagulated on boiling.	3.6	3.1	3.6	3.0	0.0	0.0	0.3	1.3
IIA 17	Mc tritis.	"	"	"	4.2	3.2	3.7	3.9	0.0	0.0	0.3	3.0
HA 19	Abscess.	"	"	"	3.9	3.1	2.7	3.9	0.1	0.0	0.2	2.5
N 23	Nasal exudate; rhinitis.	"	"	"	4.4	3.6	3.8	4.0	0.0	0.0	1.3	3.0
In. 2	"	"	"	"	4.3	0.3	9.4	4.0	1.0	0.0	0.3	4.0
In. 3	"	S. C.	Clear.	Unchanged.	4.5	0.1	4.1	4.3	0.1	0.1	0.3	3.5
In. 5	"	"	Turbid.	"	4.4	0.1	4.0	4.4	0.0	0.0	0.3	3.5
In. 6	Pharynx;	M. C.	"	"	4.4	0.1	3.9	4.3	0.0	0.0	0.3	3.0
In. 9	"	S. C.	"	"	4.3	0.0	4.4	4.5	0.2	0.2	4.1	5.2
In. 10	"	"	"	"	4.7	0.2	4.4	4.7	0.0	0.0	0.3	9.1
In. 11	Exudate from eye;	"	Turbid.	"	4.5	0.4	1.4	4.0	0.0	0.0	0.3	4.0
In. 12	"	"	"	"	4.4	0.1	4.1	4.5	0.0	1.0	0.3	4.5
In. 73	"	M. C.	Clear.	"	4.8	0.4	5.4	7.0	0.0	0.0	1.4	2.2
In. 86	"	L. C.	"	"	4.1	0.3	6.4	6.0	0.0	1.0	0.3	1.3
Str. 6	Nasal exudate; strangles.	S. C.	"	"	4.2	0.4	0.3	8.0	0.0	0.0	0.3	9.2
Str. 9	"	M. C.	"	"	4.2	0.3	7.4	4.0	0.0	0.0	0.3	5.3
Str. 12	"	"	"	"	4.0	0.3	7.4	1.0	1.0	0.0	0.3	2.3
Str. 16	Abscess;	"	"	"	3.9	0.1	3.1	3.7	0.1	0.2	2.3	6.3
Str. 18	"	S. C.	"	"	3.1	0.3	5.3	2.0	0.0	0.0	0.2	6.3
Str. 23	"	Pairs and S. C.	"	"	3.8	0.1	3.8	4.0	0.1	0.1	0.3	0.3
HA 8	"	"	"	"	3.4	0.1	3.4	3.5	0.0	0.0	0.3	5.2
N 23A	Nasal exudate; rhinitis.	M. C.	Clear.	"	4.6	0.1	4.6	4.0	1.0	1.0	1.4	6.3
P 70	Pharyngitis.	L. C.	Turbid.	"	4.8	0.4	3.4	8.0	1.0	1.0	0.3	4.3

cause septicemia but usually produces severe febrile disturbances or causes localization in joints. Arthritis is the most frequent result of intravenous injections.

The colonies of *Streptococcus pyogenes* are round, slightly raised, translucent, and rarely exceed 1.5 mm. in diameter. The hemolytic area is of the beta type and is pronounced about both the surface and deep colonies. The colonies of *Streptococcus equi* are larger, more opaque, sharply raised, and glistening. Hemolytic zones develop about both the surface and deep colonies. On horse blood agar slants the non-lactose-fermenting type produces a much thicker, glistening, opaque growth and resembles *Streptococcus mucosus*. The other forms a more delicate, thin dry film.

Preparations from the peritoneal exudate of mice dying as the result of inoculation, stained by Gram's method and counterstained with safranine, show both types grouped in pairs and short chains. A well developed capsular substance can usually be demonstrated about the cocci. In films made from the pus of infected horses *Streptococcus equi* usually appears as diplococci and chains of 10 or 12. In one instance extremely long chains were observed. *Streptococcus pyogenes* frequently occurs in pairs and in chains of 6 or 8.

#### DISCUSSION.

The true etiological relationship of streptococci to both strangles and influenza is still in doubt. There can be no question, however, that they play an important part in both diseases. *Streptococcus pyogenes* has been found more frequently in horses suffering from influenza than *Streptococcus equi*, although the latter species has been isolated from four typical cases. In strangles the opposite holds true, but in certain instances *Streptococcus pyogenes* has been isolated in pure culture. It is possible that nasal and pharyngeal infections depend, to a considerable degree, on a lowered resistance. Such a condition may exist either as the result of a distinct primary infection due to another organism or virus, or to a number of external causes. The proportion of infected animals following a journey appears to depend somewhat on atmospheric conditions, diminished rations, overcrowding, and the length of the journey. One fact which points to a cause or causes other than the streptococcus is the frequency



with which apparently normal horses carry virulent *Streptococcus pyogenes* on the nasal mucosa and in the throat. Such horses in this district are purchased by farmers who immediately house them with other horses on their farms. Influenza rarely develops among the resident horses. It is not uncommon, however, for an aged horse reared in the East to develop influenza or strangles after exposure to these diseases in sales stables.

It is becoming a common practice to attempt to immunize horses with killed cultures of streptococci before shipment. From this investigation it seems that these vaccines should contain both the lactose-fermenting *Streptococcus pyogenes* and the non-lactose-fermenting *Streptococcus equi*.

The horse streptococci, especially *Streptococcus pyogenes*, resemble those of human and bovine types to a certain extent. The cow strains, from mastitis, are easily separated from those of the horse by their low pathogenicity for mice and rabbits and their ability to coagulate milk: Bovine streptococci from mastitis rarely produce well marked areas of hemolysis about the surface colonies. Hemolytic zones usually develop about the surface colonies of the pathogenic human and equine strains. The pathogenic horse streptococci closely resemble those from human diseases in pathogenicity for rabbits and mice and in their hemolytic properties. Avery and Cullen (21) have shown that the hydrogen ion concentration of the bovine strains grown in dextrose broth ranges between 4.3 and 4.5, while that of the human strains is 5.0 to 5.2. These differences have held true when measured with 0.05 N sodium hydroxide. The acid production of the bovine strains from mastitis has averaged 4.4 per cent of normal acid, of those pathogenic for the horse the net production is a little lower, 4.1 per cent. The human strains, however, are considerably lower, 2.6 per cent. Thus acid production appears to serve as a ready means of differentiation between the pathogenic animal and human strains.

#### SUMMARY.

The lower nasal mucosa and the pharynx of thirty eastern and twenty-three western horses have been examined for streptococci.

Eight of the eastern horses carried non-hemolytic streptococci on the nasal mucosa. From the pharynx of six, non-hemolytic strepto-

cocci were cultivated. The throats of eighteen contained strains of the hemolytic type. The nasal mucosa of the eastern horses failed to show hemolytic streptococci.

Eight western horses carried non-hemolytic streptococci in the nasal passage; eight also harbored the hemolytic type. Twenty-two strains were isolated from the pharynx. Eleven were hemolytic.

Among all the non-hemolytic nasal strains those capable of fermenting mannite predominate. Those of the non-hemolytic types from the pharynx of both classes of horses may or may not ferment lactose but all do ferment either raffinose or inulin. In no instance have any of the non-hemolytic types proved pathogenic for mice.

The hemolytic strains from the nasal mucosa of the western horses were all of the *Streptococcus pyogenes* type. They were pathogenic for mice and rabbits. One strain from the pharynx of an eastern horse and eight from the throats of the western horses were of the same species. All the others corresponded closely in their fermentation reactions with non-hemolytic streptococci from the same region.

The streptococci from pathological sources were all hemolytic. They have fallen into two groups; the larger group (*Streptococcus pyogenes*) produced acid in dextrose, lactose, saccharose, maltose, milk, and salicin but failed to change the reaction of broth containing raffinose, inulin, or mannite. The streptococci of the smaller group (*Streptococcus equi*) differ only in their inability to ferment lactose or acidulate milk. Both types are pathogenic for mice. Rabbits are usually more resistant.

*Streptococcus pyogenes* has been isolated from eighteen of twenty-two cases of influenza, three of six cases of strangles, and from eight of nine abscesses. *Streptococcus equi* was observed in four horses suffering from influenza and five others affected with strangles. This species was also found in an abscess and associated with both rhinitis and pharyngitis.

I am indebted to Dr. W. J. Lentz of the Veterinary Department of the University of Pennsylvania for considerable material from abscesses, and to Dr. P. J. Runyon of Freehold, N. J., through whom a great portion of the material was obtained. Dr. R. B. Little of

this department assisted in collecting and also obtained much material.

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#### EXPLANATION OF PLATE 12.

FIG. 1. Plate Culture P 40. Hemolysis Type xa; 2 per cent veal infusion agar, 12 cc., defibrinated horse blood, 1 cc.; incubation 24 hours at 38°C. The inner clearer zone surrounded by the larger area of partial hemolysis is shown.

FIG. 2. A deep colony from the same culture. Note the aggregates of unhemolyzed cells in both zones.  $\times 60$ .

FIG. 3. Plate Culture P 57. Hemolysis Type x; media and incubation periods identical with those described under Fig. 1. The hemolytic area is more or less hazy due to a partial dissolution of the red cells.

FIG. 4. A deep colony of Culture P 57. Clumps of red cells are visible throughout the area of hemolysis.  $\times 60$ .



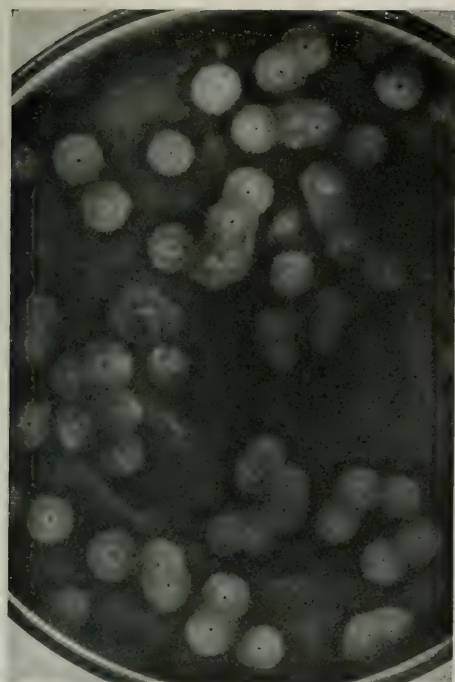


FIG. 1.

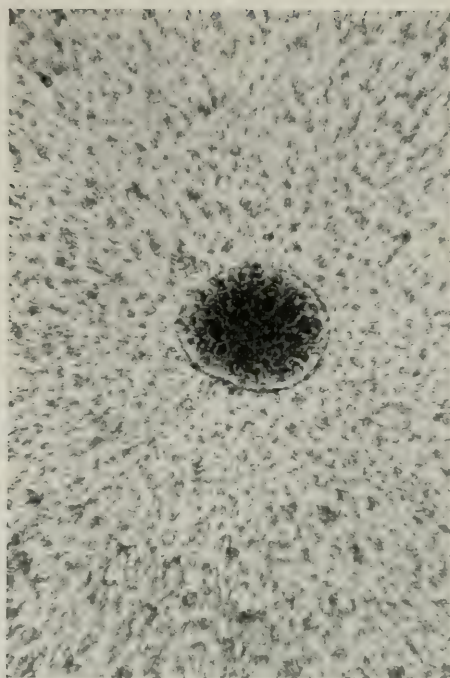


FIG. 2.

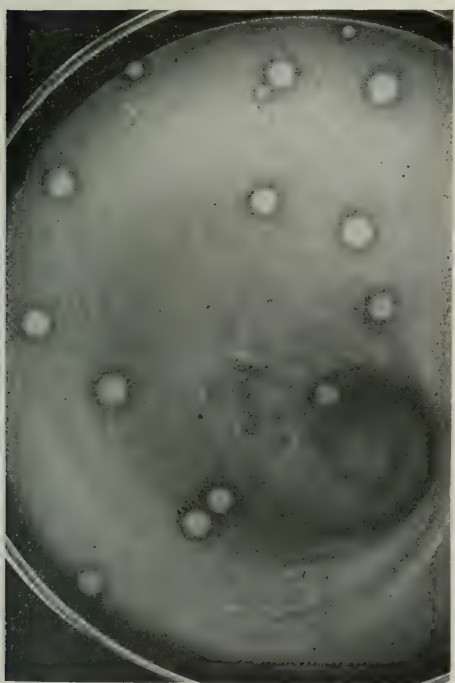


FIG. 3.

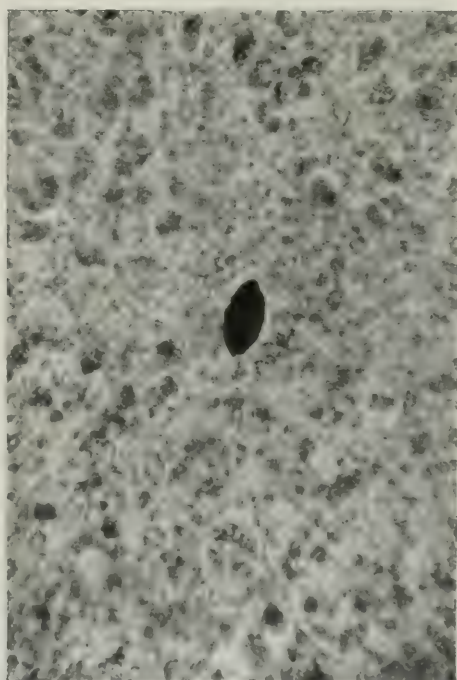


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